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TENTACLE DEVELOPMENT AND REGENERATION IN THE ANTHOMEDUSA *POLYORCHIS PENICILLATUS* AND THE INVOLVEMENT OF THE RF-AMIDE IMMUNOREACTIVE NEURONS

BY

© NANCY ALINE MCFADDEN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

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ABSTRACT

Tentacle development, and the coincident development of the RFamide-like (arg-phe amide-like) immunoreactive neurons in the hydromedusa *Polyorchis penicillatus* was described. Stages of tentacle development were identified. In Stage 0, no bud was visible on the basis of external morphology, however it was known that a bud would form in thisintertentacular region. There was no RFamide immunoreactivity in the region where the bud would form, other than the diffuse plexus of the intertentacular region. In Stage 1, the ectoderm thickened in the region of the future tentacle, forming a small bud at the bell margin. There were typically one to two immunofluorescent cell bodies in the bud, amidst the intertentacular network. Within one day, in Stage 2, endoderm from the ring canal outpocketed into the ectodermal bud and formed the tentacle canal. Typically, there were 12 - 16 immunofluorescent cell bodies at this stage, which were arranged at the tip of the bud in one or two cup-like arrangements around the endodermal canal and extended neurites to the outer nerve-ring. After approximately 5-6 days the ocellus, or eyespot formed on the aboral surface of the tentacle bud. The formation of the ocellus characterized Stage 3. A few large cells became immunoreactive at the bud apex, before the pigment cells of the ocellus were visible. These cells were distinguishable from the cup-like arrangements of cell bodies, which were more proximal to the bell margin, and which remained at the base of the tentacle. The number of immunoreactive ocellar cells increased during the time that the ocellus formed. The immunoreactive ocellar cells lay in a semi-circle around the periphery of the ocellus itself, and extended neurites in the ocellar nerves to the bell margin. Once the ocellus formed, the bud began to elongate to form a tentacle rudiment (a stage 4 bud). At this time the RFamide ectodermal nerve-net of bi- and tri-polar neurons became visible in the tentacle. The tentacle rudiment appeared to function physiologically as a mature tentacle. The development of the RFamide-positve neurons

during development was indirectly compared to the development of the 'B' system neurons using extracellular recording techniques. It was found that the 'B' system formed later than the RFamide-positive system in the bud.

Tentacle regeneration in culture was limited to younger animals (<24 tentacles). When there was tentacle regeneration, it occurred with high fidelity both in terms of gross morphology, the the morphology of the RFamide-like immunoreactive neurons. Tentacle development and regeneration in animals which had been depleted of the multipotent stem cell, the interstitial cell, was minimal and was restricted to tentacle elongation only. The only RFamide-positive neurons present in the developing or regenerating tentacles after interstitial cell depletion were those immunoreactive cells of the tentacle ectodermal nerve-net which formed in the elongating tentacle.

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TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION	1
Rationale for the study	1
Literature cited	9
II. Development of tentacles in the anthomedusa *Polyorchis penicillatus*, and the structure of the RFamide-like system of neurons which innervates them	15
Introduction	15
Methods and Materials	24
Results	28
Discussion	48
Literature cited	59
III. Growth and regeneration of tentacles of *Polyorchis penicillatus* in a culture system	63
Introduction	63
Methods and Materials	66
Results	73
Discussion	83
Literature cited	92
IV. CONCLUSION	97
V. APPENDICES	102
I- Production of Monoclonal antibodies against Polyorchis tissue	102
II- Rate of Regeneration in Culture	107
III- Immunocytochemistry against RFamide peptides in Polyorchis tissue	109
Literature Cited	112

LIST OF TABLES

TABLE		PAGE
Table 3.1	Summary of Extirpation Experiments	67
Table 3.2	Interstitial Cell Population after Hydroxyurea treatment	70
Table 3.3	Rate of Development in Culture	74

LIST OF FIGURES

FIGURE		PAGE
2.1	Schematic diagram of *Polyorchis* with a portion of the bell removed to show its general features	17
2.2	Schematic diagram to show the distribution of the different parts of the nervous system	19
2.3	Tentacle placement during development	23
2.4	Line drawing showing the stages of tentacle development	34
2.5	Extracellular recordings of the 'B' system in tentacle buds of different stages	47
2.6	Schematic diagram illustrating a possible connection of the 'B' system to the RFamide system during tentacle development	56
3.1	Bell Diameter as a Function of Tentacle Number	75

LIST OF PLATES

PLATE		PAGE
2.1	The morphological characteristics of young and mature medusae	30
2.2	Stages of tentacle development in *Polyorchis penicillatus*.	32
2.3	The RFamide-like immunoreactivity of the mature tentacle	37
2.4	RFamide-like immunoreactivity of the tentacle bud stages	41
2.5	The RFamide-like immunoreactivity of the developing tentacle at Stage 3, viewed from the INR side	44
3.1	Mature tentacles lacking ocellar pigment, but showing normal RFamide-like immunoreactivity	78
3.2	The RFamide-like immunoreactivity of a regenerated tentacle	81
A.1	Staining patterns of monoclonal antibodies developed against *Polyorchis* tissue	105

I. INTRODUCTION

Rationale for the study

It is generally accepted that communication between neurons, or from a neuron to an effector cell, except in the case of electrical communication, involves the release of chemical neurotransmitters at the synapse. Before 1960, the amines, acetylcholine, norepinephrine, and serotonin were the only recognized neurotransmitters. Amino acids such as gamma aminobutyric acid (GABA), glutamic acid, aspartic acid, and glycine were then added to the list of putative neurotransmitters (Synder, 1984). The discovery that peptides might also be used for signalling between neurons was a dramatic development in neuroscience. Substance P was the first neuropeptide to be proposed as a neurotransmitter (Bradford, 1986, see review). Now at least 100 neuropeptides have been identified as having possible chemical messenger function (Eipper *et al,* 1986).

Although it has been documented that many peptides, such as LHRH (lutenising hormone-releasing hormone) and opioids, function as neurotransmitters, the physiological functions of other neuropeptides remain unclear, despite much experimental investigation (Zachary *et al.*, 1987, see review). There is a rapidly growing number of neuropeptide families which exhibit both structural diversity, and a wide spectrum of biological effects. Many of these neuropeptides seem to perform modulatory or regulatory roles that do not easily fit into a conservative model of a transmitter. Some neuropeptides demonstrate mitogenic and morphogenic effects, whether they show neurotransmitter capabilities or not. Substance P, for instance, is a known neurotransmitter, but it has also been shown to have a direct growth promoting effect on smooth muscle cells and human skin fibroblasts (Nilsson *et al.*, 1985; Payan, 1985). Similarly, other evidence indirectly suggests that the

release of tachykinins from sensory nerves in the skin, joints, and other peripheral tissues might function as mediators of local inflammatory and wound healing responses (Zachary *et al.*, 1987). In the planarian *Dugesia tigrina*, Substance P has been shown to stimulate cellular proliferation and differentiation in both intact and regenerating animals (Salo and Baguna, 1986). Other neuropeptides which show mitogenic activity are bombesin (Rozengurt and Sinnett-Smith, 1983); vasopressin (Rozengurt *et al.*, 1979); gastrin (Kobori, *et al.*, 1982); and ß-endorphins (Gilman *et al.*, 1982). Given the current controversies regarding the role(s) of neuropeptides, it is valuable to further our understanding of the function of neuropeptides by examining the role peptides play in the most simply organized animals which possess a nervous system, the cnidarians.

There is both morphological and physiological evidence of chemical transmission both within (scyphozoans, anthozoans) and between (hydrozoans) neuronal networks, and onto effector cells, in cnidarians (Jha and Mackie, 1967; Westfall, 1973a,b; Singla, 1978a,b; Spencer, 1978; 1982; Roberts and Mackie, 1980; Spencer and Arkett, 1984; Anderson, 1985; Quaglia and Grasso, 1986). Excitatory postsynaptic potentials (EPSPs) characteristic of chemical transmission have been observed at cnidarian synapses. For instance, Anderson and Mackie (1977) demonstrated that tentacle contractions and input from ocelli caused EPSPs in swim motor neurons of *Polyorchis penicillatus* . Although no neurotransmitter has been conclusively identified in cnidarians, physiological studies have demonstrated that post-synaptic potentials can be inhibited by excess magnesium or calcium depletion, supporting the notion of Ca^{++} mediated transmission (McFarlane, 1973; Spencer, 1978; Satterlie, 1979; Martin and Spencer, 1983; Spencer and Arkett, 1984). In ultrastructural studies, it has been shown that all classes of cnidarians possess dense-cored, electron-dense, and electron-lucent vesicles in close association with pre- or postsynaptic membrane specializations,which are structures traditionally associated with chemical synapses (Jha and Mackie, 1967; Westfall, 1970; 1973a,b; Spencer, 1979). Also, synaptic

delays of 0.9-7ms, which are characteristic of chemical transmission, have been seen in several cnidarian preparations (Roberts and Mackie, 1980; Spencer, 1982; Anderson, 1985). However, certain aspects of chemical neurotransmission in cnidarians, for instance the identity of transmitters, is poorly understood.

Studies on both the histochemical localization and physiological actions of adrenaline and noradrenaline in the Cnidaria have been inconclusive, as have studies on 5-HT (5 hydroxy-tryptamine) (Martin and Spencer, 1983). Dopamine is present in the nerve-rich tissue of *Polyorchis penicillatus* (Chung *et al.*, 1989). Catecholamines have also been demonstrated in *Hydra* sp., and there is some physiological evidence of catecholamines in the anthozoan, *Renilla köllikeri* (Carlyle, 1969a,b; Lenique *et al.*, 1977; Anctil *et al.*, 1982; Venturini *et al.*, 1984; Carlberg and Rosengren, 1985; De Waele *et al.*, 1987) and in the hydrozoan *Halocordyle disticha* (Kolberg and Martin, 1988) but their bioactivity is not well understood (Anctil *et al.*, 1982).

Immunocytochemical methods indicate the presence of compounds similar in structure to various neuropeptides (oxytocin, bombesin, cholecystokinin/gastrin, neurotensin, FMRFamide (phe-met-arg-phe- amide) and substance P) in different populations of neurons in *Hydra* (Grimmelikhuijzen *et al.*, 1980; 1981a,b,c; 1982; Grimmelikhuijzen, 1985). Fewer neuropeptides have been found in hydromedusae as compared to *Hydra*, but the antisera to a family of neuropeptides with an RFamide (Arg Phe amide) carboxy terminus, originally isolated as FMRF amide (Phe Met Arg Phe amide) from the clam *Macrocallista nimbosa* (Greenberg and Price, 1979), is consistently immunoreactive in subsets of the nervous system in the medusae of *Polyorchis penicillatus* (Anthomedusae); *Aglantha digitale* (Trachymedusae); *Proboscidactyla flavicirrata*; *Gonionemus vertens*; *Eperetmus typus* (Limnomedusae); the leptomedusae *Phialidium gregarium* and *Aequoria victoria*; and siphonophores (Mackie and Stell, 1984; Mackie *et al.*, 1985; Grimmmelikhuijzen *et al.*, 1986).

Immunoreactivity to the RFamides has been demonstrated in invertebrate systems such as molluscs (Greenberg and Price, 1979; Cottrell *et al.*, 1983b; Schaefer *et al.*, 1985), *Limulus* (Watson and Groome, 1984); the leech (Kuhlman and Calabrese, 1985), as well as some insects (Walther *et al.*, 1984; Carroll *et al.*, 1986). Immunoreactivity to the RFamides is also found in vertebrates such as goldfish (Stell *et al.*, 1984; Muske *et al.*, 1987); mice (Boer *et al.*, 1980) and rats (Chronwall *et al.*, 1984; O'Donohue *et al.*, 1984). Most physiological studies on the role of FMRFamide, and other members of this peptide family, have been undertaken using various molluscs. FMRFamide has potent pharmacological actions on a range of cardiac and non-cardiac muscles and neurons of different species of molluscs (Greenberg and Price, 1983; Price, 1986). This peptide is generally excitatory on cardiac muscle, but in some species it is inhibitory (Painter and Greenberg, 1982). FMRFamide induces sustained contractions in molluscan smooth muscle, for example, in the radula protractor muscle of the whelk *Buscycon contrarium* and the anterior byssus retractor muscle of the mussel *Mytilus edulis* (Price and Greenberg, 1977; Greenberg and Price, 1979; Painter, 1982). In the tentacle retractor muscle of *Helix aspersa*, FMRFamide produces a prolonged contraction on which phasic rhythmical contractions may be superimposed (Cottrell *et al.*, 1983a,b). In *Aplysia*, FMRFamide causes an increase in the amplitude of the gill withdrawal reflex which is mediated through increased levels of cAMP (Weiss, *et al.*, 1984). FMRFamide also has a variety of effects on gastropod neurons such as sodium-mediated depolarization, potassium-mediated hyperpolarization (Cottrell, 1983; Murphy *et al.*, 1985). In a given neuron, more than one response may occur, so that the response may be due, for example, to the combination of an increase in Na^+ conductance and an increase in K^+conductance (Cottrell *et al.*, 1984). In molluscs then, the physiological sites of action of FMRFamide are at the molluscan myocardium, and as a neurotransmitter, neuromodulator, or

neurosecretory substance at particular neuromuscular junctions and neuro-neuronal synapses (Cottrell *et al.*, 1983b).

Although evidence is accumulating in molluscs and other animals that FMRFamide and structurally related peptides (tetrapeptides: FLRFamide; FIRFamide; pQGRFamide; pentapeptides: FMLRFamide; PFLRFamide; heptapeptides: pQDPFLRFamide; YGGFMRFamide) are neurotransmitter substances (Price and Greenberg, 1977; Boer *et al.*, 1980; Cottrell, 1982; Cottrell *et al.*, 1983a,b; Greenberg and Price, 1983; Weiss *et al.*, 1984; Price *et al.*, 1985; Cottrell and Davies, 1986; Cottrell *et al.*, 1986) their functions in cnidarians remain largely unknown. Despite numerous studies which demonstrate immunoreactivity to RFamides in cnidarians, only a few studies have found physiological effects of RFamides in cnidarians. In the sea pansy *Renilla köllikeri*, FMRFamide and RFamide were found to cause rachidial contractions and potentiate activities controlled by the through-conducting nerve-net (Anctil, 1987). No neuronal effects were found and it was concluded that the peptides had a direct effect on the muscle (Anctil, 1987). In the sea anemone *Calliactis parasitica*, endodermal application of Antho-RFamide (<Glu-Gly-Arg-Phe-NH_2) caused a long-lasting increase in tone, contraction frequency, and contraction amplitude in several slow muscle groups (M^{c}Farlane *et al.*, 1987). The peptide had no effect on the through-conducting nerve-net, and it was concluded that the peptide might be neuro-excitatory, but also probably involved a direct excitation of the muscles (M^{c}Farlane *et al.*, 1987). In the hydromedusa *Polyorchis penicillatus*, FMRFamide and related peptides (RFamide, LPLRFamide, and pEGRFamide) were found to cause an initial transitory hyperpolarization of the swim motor neurons (SMNs) in the inner nerve-ring of the bell margin, followed by a long-duration depolarization (Spencer, 1988). The initial hyperpolarization was sometimes absent (Spencer, 1988). The endogenous, active peptides in these cnidarians are almost certainly not FMRFamide or RFamide, although the native peptides do share the carboxy terminus, RFamide, which is required for biological

activity and immunoreactivity (Painter *et al.*, 1982; Walther *et al.*, 1984; Grimmelikhuijzen and Graff, 1986). A native neuropeptide, Pol-RF amide, which has been isolated from *Polyorchis penicillatus*, and sequenced, also has the RFamide 'message'. Pol-RFamide does not elicit any detectable response in impaled neurons *in vivo* , except at very high concentrations (Spencer, pers. comm.). It is possible that the technique of application was not effective, or that the epithelial cells ensheathing the neurons present a barrier for the penetration of the peptide. In cultured SMNs, Pol-RFamide causes a long duration hyperpolarization, however, the concentrations used were quite high (10^{-4} to 10^{-8}M) (Spencer, pers. comm.). The effect of Pol-RFamide on cultured neurons is specific but not necessarily physiological. The observation that the peptide Pol-RF amide (<Glu-Leu-Leu-Gly-Gly-Arg-Phe-NH_2), can be localized to neurons immunohistochemically in *Polyorchis*, but is not known to be active in the animal's behaviour, does not preclude the possibility that these peptides are involved in the neurotransmission mechanism. Recently however, immunogold labelling of RFamide-like peptides in both the outer and inner nerve-rings of *Polyorchis penicillatus* localized the peptide to non-synaptic dense-cored vesicles (Singla, unpublished data). Similarly, in *Hydra*, RFamide has been localized immunocytochemically to dense-cored vesicles (Koizumi, *et al.*, 1989). In *Velella*, however, RFamide-like immunoreactivity is localized to a sensory network which lacks dense-cored vesicles (Mackie, *et al.*, 1988). The possibility of multiple functions for this peptide family such as neurotransmission, neuromodulation, neurohormonal or morphogenic action cannot be ignored. These peptides may act as morphogenic factors, like the 'head activator' of Bodenmüller and Schaller (1981) or, as claimed for Substance P in planarians by Lender (1974), these peptides may be active in the control of regenerative phenomena. Indeed, it has been demonstrated in *Halocordyle disticha*, that there is a developmental correlation between the time of expression of the RFamide phenotype, and metamorphosis of the planula larva into an adult polyp (Martin, 1988). It could be that

RFamides act as morphogens in the same way that classical transmitters such as 5HT have been shown to influence specific neuronal outgrowth, and neuropeptides such as bombesin and substance P have been shown to influence growth and regeneration (Haydon *et al.*, 1984; Zachary *et al.*, 1987). Therefore, for the following reasons, RFamide might be important during the development of tentacles in *Polyorchis*: i) many neuropeptides are known to be morphogens and mitogens; ii) RFamides appear to be the dominant neuropeptides present in the nervous systems of cnidarians; and iii) the phenotypic expression of RFamides has been shown to appear briefly in another hydrozoan, *Halocordyle*, at a critical time during development (metamorphosis) and subsequently disappear.

In the first study (Chapter 2), the major objectives are: i) to describe the morphology of a developing tentacle in the hydrozoan *Polyorchis penicillatus* ; ii) to determine the extent and nature of RFamide-like immunoreactivity (RFIR) throughout tentacle development in *Polyorchis penicillatus* . The association of RFIR with specific anatomical features during development may allow inferences as to possible function of the neuropeptide; iii) to correlate the development of the RFIR components with previously identified neuronal systems in hydromedusae. The second study (Chapter 3) is a comparative study of ontogeny and regeneration of the tentacle in *Polyorchis penicillatus*. The main objectives of this study are: i) to compare the development of the regenerating tentacle, and its RFamide neuronal system with the development of the tentacle and its RFamide neuronal system during ontogeny; and ii) to examine the ontogenic and the regenerative potential of the tentacle and of the RFamide neuronal system, in the absence of neuronal precursors. These objectives examine the role neurons may play in the development and regeneration of the tentacle. Chapter 4 consists of concluding remarks. Appendices I and III provide details on work that I completed on developing monoclonal antibodies to *Polyorchis*

tissue, and on immunocytochemical localization of RFamide in *Polyorchis* at the E.M. level.

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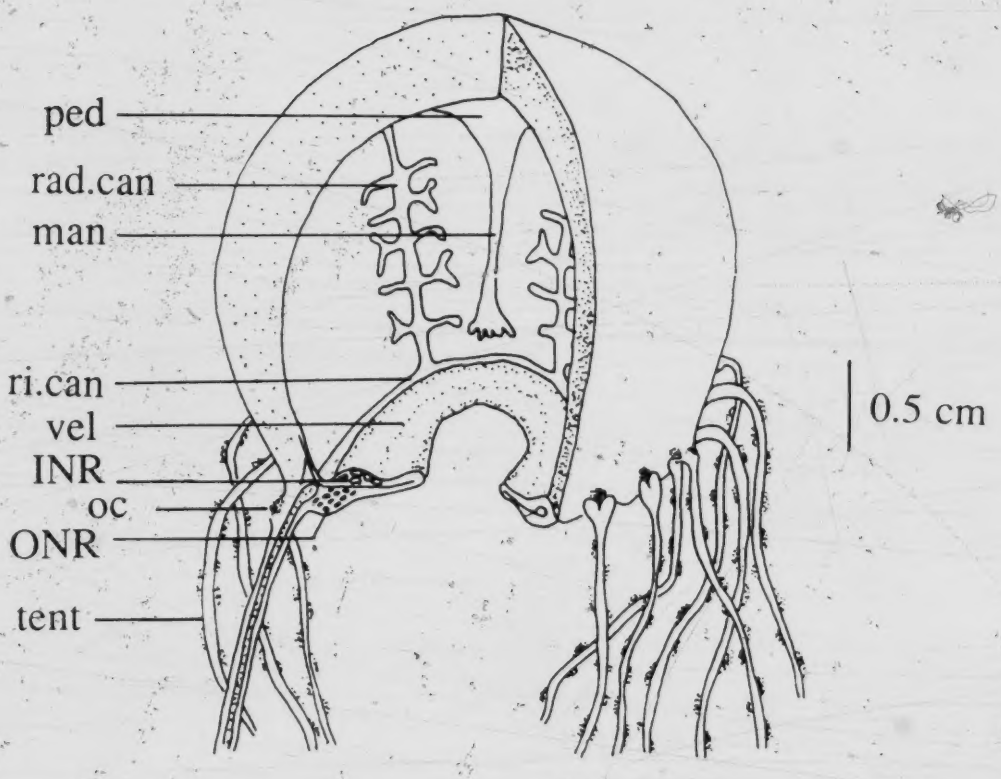
II. Development of tentacles in the anthomedusa *Polyorchis penicillatus*, and the structure of the RFamide-like system of neurons which innervates them.

Introduction

The gross morphology of the nervous system of *Polyorchis penicillatus* is typical of anthomedusae (Figure 2.1). The nervous system consists of several components. In the tentacles, manubrium, and the gonads there is a peripheral, diffuse plexus in the ectoderm (Figure 2.2a). There is also a nerve-plexus which runs throughout the endodermal ring and radial canals (Figure 2.2b) (Singla, 1978; Spencer, 1979). The axons of this plexus run parallel to the long axis of the canal and extend into the lateral arms of the radial canals (Singla, 1978; Spencer, 1979). There are no apparent connections between the ectodermal and endodermal nervous systems (Singla, 1978; Spencer, 1979). At the bell margin there is a condensed 'central nervous system' of two nerve-rings, the inner nerve-ring (INR) and the outer nerve-ring (ONR), which run parallel to the velum (Figure 2.1) (Singla, 1978; Spencer, 1979). The INR is subumbrellar and has a mostly motor function (Figure 2.2c). The swim motor neurons (SMNs) are a group of 'giant' neurons (approx. 20x150 μm) which lie within the INR as an anastomosing network. These SMNs are electrically coupled via gap junctions to one another (Spencer and Satterlie, 1980). The SMNs directly control swimming contractions through neuromuscular synapses with swimming muscle cells at the bell margin and up the radii (Singla, 1978; Spencer, 1979). In addition to the SMNs there are as many as 500 neuronal profiles making up the INR which are grouped around and between the giant axons (Spencer, 1979). Along each per-radius, two distinct

Figure 2.1 Schematic diagram of *Polyorchis* with a portion of the bell removed to show its general features, and the orientation of the nerve rings on either side of the velum to the bell and to the tentacles. ped, peduncle; man, manubrium; ri.can, ring canal; rad.can, radial canal; vel, velum; INR, inner nerve ring; oc, ocellus; ONR, outer nerve ring; tent, tentacle.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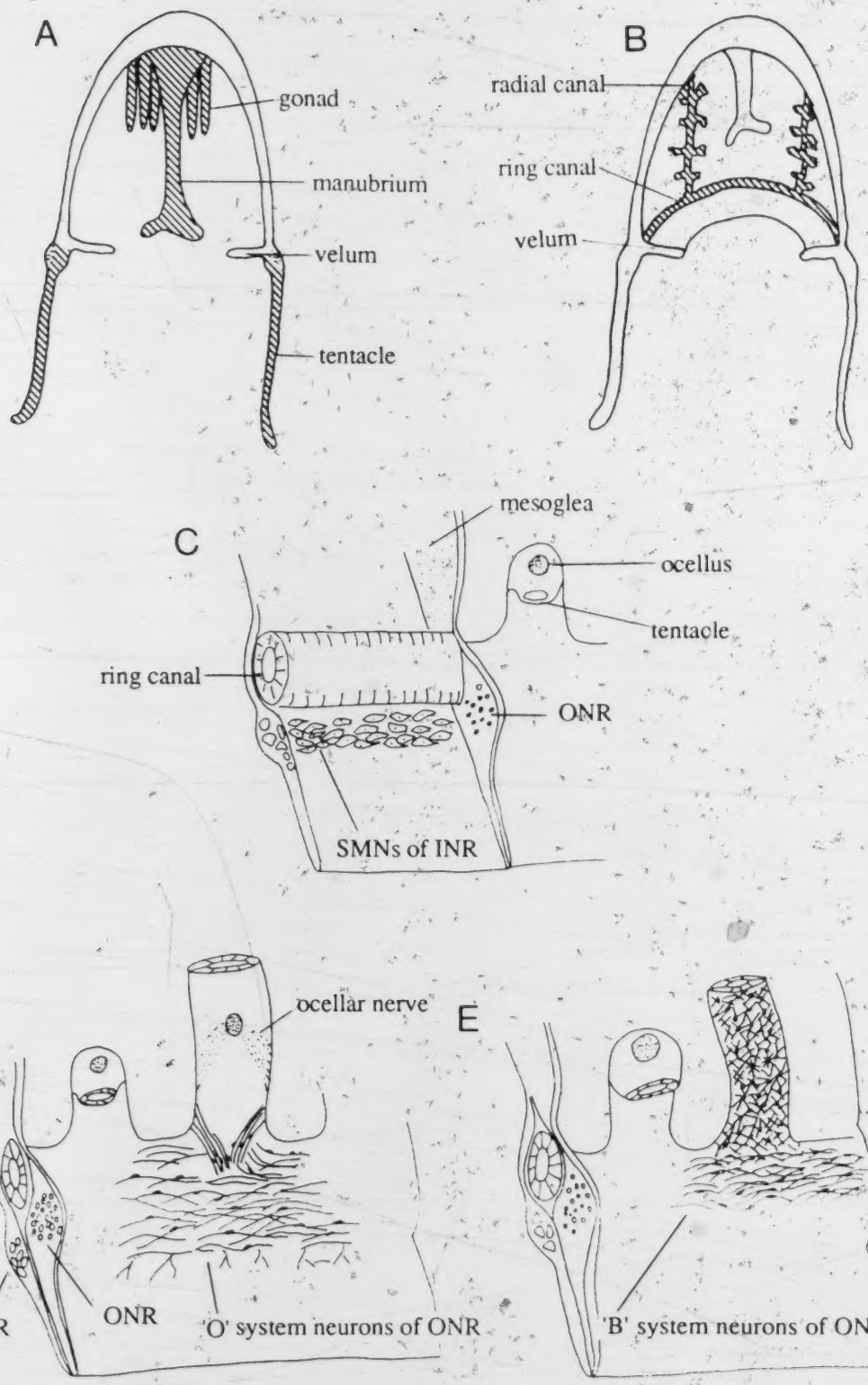
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manubrium
velum
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radial canal
ring canal
velum
C
mesoglea
ocellus
tentacle
ring canal
ONR
SMNs of INR
D
ocellar nerve
INR
ONR
'O' system neurons of ONR
E
'B' system neurons of ONR

bundles of axons run along either side of the radial muscle band from the SMN network of the INR to the manubrium (Singla, 1978; Spencer, 1979). The fate of these radial nerves beyond the manubrium is not known (Spencer, 1979).

The INR also has connections with the ONR. The ONR consists of smaller diameter neurons on the exumbrellar side of the velum. Two neuronal networks of the ONR have been identified both morphologically and physiologically (Figure 2.2d,e) (Spencer and Arkett, 1984). The 'O' (oscillator) system is an electrically coupled neuronal system (Figure 2.2d) (Spencer and Arkett, 1984; Arkett and Spencer, 1986a,b). The non-spiking 'O' system shows regular, spontaneous, membrane potential oscillations with amplitudes of approximately 20mV in lighted conditions (Spencer and Arkett, 1984; Arkett and Spencer, 1986a). The 'O' system is photosensitive and shows graded hyperpolarizations in response to decrements in light intensity, which interrupt the oscillations, and are maintained for some time (Arkett and Spencer,1986b). An increase in light intensity causes a rapid depolarization in the 'O' system and then a resumption of the former oscillations (Spencer and Arkett, 1986a,b). The second identified neuronal network of the ONR, the 'B' system, or bursting system (Figure 2.2e) is also a system of electrically coupled neurons. 'B' system action potentials, when a shadow is cast upon the marginal regions, produce unitary EPSPs in the swim motor neurons and in the epithelial cells,overlying the ONR (Spencer, 1981; Spencer and Arkett, 1984). The 'B' system, is so named because it is characterized by a regular firing of up to six spikes per burst, which can be recorded intracellularly in response to a rapid reduction in light intensity (Spencer and Arkett, 1984). Bursts also occur spontaneously, but only in bursts of up to three spikes. The 'B' system controls simultaneous rhythmical tentacle contractions (Spencer, 1978; Spencer and Arkett, 1984).

In addition to these physiologically identified systems (SMNs, 'B', and 'O' systems), the recent advent of immunofluorescent techniques, and the discovery that

antisera to a family of neuropeptides with an RFamide C-terminus is consistently immunoreactive in subsets of neurons in *Polyorchis*, has allowed visualization of another major portion of the nervous system (the RFIR system), which was previously not known in *Polyorchis* (Grimmelikhuijzen and Spencer, 1984). The tentacles of *Polyorchis* are innervated by these RFamide neurons, as well as by the previously identified systems of neurons in the ONR (The 'O' and 'B' systems). The distribution of the RFamide system in the mature tentacle is similar to that of the 'O' and 'B' systems, and the possibility exists that either the 'O' or the 'B' system might be immunoreactive to RFamide (Grimmelikhuijzen and Spencer, 1984).

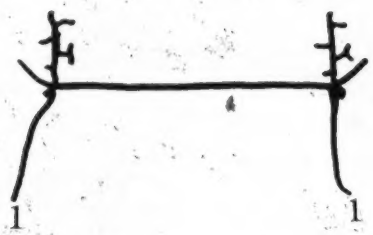
Skogsberg (1948) noted that the addition of subsequent tentacles to the bell margin during medusa development was very specific. The second whorl of tentacles were always located interradially, or one tentacle developed between each of the original tentacles. After eight tentacles were formed, the tentacles increased by multiples of two in specific locations within each quadrant (Figure 2.3). A tentacular series arose with the addition of subsequent tentacles, which was, invariably:

4 - 8 - 16 - 24 - 32 - 40 - 48 - 56 - 64 - and so on (Figure 2.3).

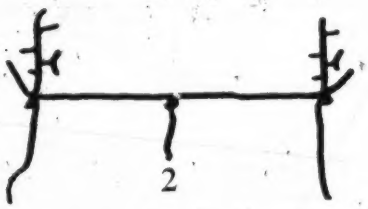
Except for Skogsberg's (1948) treatise on the systematics of the family Polyorchidae, no work has been done on the development of tentacles in medusae, or on the neuronal systems which innervate developing tentacles. The present study describes developing tentacles in the anthomedusa *Polyorchis penicillatus* , and the structure of the RFamide system which innervates them. The RFamide system is particularly examined because of their potential developmental role in *Polyorchis* tentacle development. As well, the development of the RFIR component of the nervous system in the tentacle is correlated to the development of the 'B' systems in tentacles.

Figure 2.3 **A**. Schematic drawing illustrating the placement of tentacles as they are added to one quadrant of the bell margin during the development of the medusa. After budding, a single tentacle is added per quadrant. Subsequent tentacles are added in pairs to each quadrant. **B**. The specific placement of many tentacles in one quadrant of a mature medusa according to Skogsberg, 1948 (modified).

A

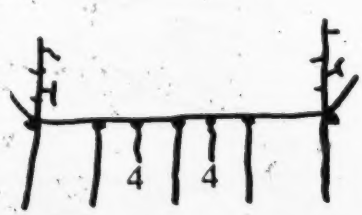


4 tentacle stage



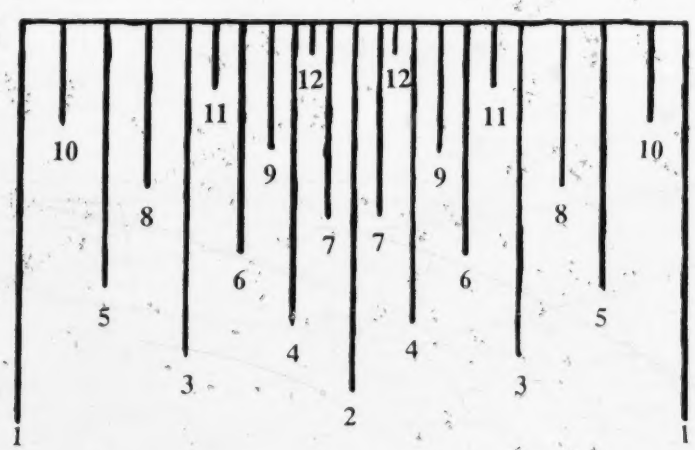
8 tentacle stage

16 tentacle stage



24 tentacle stage

B



Methods and Materials

Specimens of *Polyorchis penicillatus* (bell diameter 0.2 - 1 cm) were collected by plankton tow (120 µm net, horizontal tows) from Bamfield Inlet, Bamfield, B.C. The animals were maintained in a running seawater system at the Bamfield Marine Station.

Stages of tentacle development were identified in live specimens at 10x magnification. Criteria based on external morphology were used to subdivide the development into stages which were easily distinguished from each other. Five stages of normal budding were designated: Stage 0. No profusion of budding tissue, but bud will form in this intertentacular region according to Skogsberg's (1948) formula. Stage 1. Bud formation, ectodermal outpocketing only. Stage 2. Bud formation, ectodermal and endodermal outpocketing. Stage 3. Ocellus formation. Stage 4. Tentacle rudiment formation. Although young specimens were used exclusively (<24 tentacles), older medusae continue to form new tentacles, and show buds in all stages.

Immunohistochemistry

Medusae with tentacles at stages 0 - 4, and mature tentacles, were anesthetised in a 1:1 mixture of sea water and 0.33M Mg Cl_2, dissected, and fixed in a fresh solution of 4% paraformaldehyde in 0.1M sodium phosphate buffer, pH 7.3, at 4°C for 12 to 24 h. After fixation, the tissue was washed for 1h in phosphate buffered saline (PBS), incubated for 4h in 0.2M glycine, to quench the free aldehydes, and rinsed for 1h in PBS with 0.25% Triton-X 100 (PBS-Triton). The specimens were incubated overnight at 4°C in antiserum to RFamide (146II, kindly supplied by C. Grimmelikhuijzen) diluted 1:1000 in PBS-Triton with 0.25% human serum albumen. The next day the tissue was rinsed for 1h in PBS-

Triton, and subsequently incubated overnight in fluorescein isothiocyanate-labelled goat anti-rabbit IgG (Sigma) diluted 1:100 with PBS-Triton. The specimens were then rinsed for 1h in PBS, counterstained in a 0.5% solution of Evans blue (Merck) in PBS for 1 minute. Excess stain was removed by rinsing the tissue for 1h in PBS. The specimens were mounted on slides with the subumbrellar surface, and hence the ONR, uppermost, or the reverse, with INR uppermost, in Mowiol mounting medium (Osborn and Weber, 1982) (Mowiol 4-88, Calbiochem), modified by the addition of 0.5g 1-4- diazabicyclo (2,2,2) octane ('DABCO') per 24 ml of Mowiol solution to prevent photobleaching (Zalik, *et al.*, 1990), and viewed with a Zeiss standard microscope equipped with an epifluorescence UV lamp and accessories. An excitor filter with a 495 nm maximum transmission peak was used. Two controls using a) antisera preabsorbed with synthetic FMRFamide (100-500 µg FMRFamide/ml antisera), and b) primary antisera containing no anti-RFamide, were included in all tests. The number of buds examined in each stage is as follows: Stage 1, n=26; Stage 2, n=31; Stage 3, n=30; Stage 4, n=34.

The antiserum against RFamide used in the present study (146II) was characterized by Grimmelikhuijzen (Grimmelikhuijzen and Spencer, 1984). Antiserum 146II was obtained from rabbits immunized with synthetic RFamide (Bachem), which was coupled via carbodiimide to bovine thyroglobin (Grimmelikhuijzen and Spencer, 1984). The antiserum was treated overnight at 0°C with 2 mg/ml thyroglobin, followed by centrifugation, to remove antibodies specific for the carrier protein (Grimmelikhuijzen and Spencer, 1984). The antiserum was further characterized by overnight incubation of the diluted antiserum at 0°C with 100 µg/ml sepharose-bound RFamide, FMRFamide, and FLRFamide, followed by the removal of the solid phase through centrifugation (solid-phase absorption), which abolished all staining of 146II (Grimmelikhuijzen and Spencer, 1984). Solid-phase absorption with RFamide-related peptides, WMDFamide (cholecystokinin-(30-33)-tetrapeptide), YGGFMRF (Met-enkephalin-Arg-Phe), YVMGH-

FRWDRFG ($\sqrt{2}$-MSH), or the fragment Famide did not affect the staining by the antiserum (Grimmelikhuijzen and Spencer, 1984). Solid-phase absorption of the antiserum with peptides unrelated to RF-amide, such as gastrin-releasing peptide, neurotensin, oxytocin, substance P, and vasopressin did not influence the staining patterns of antiserum 146II (Grimmelikhuijzen and Spencer, 1984).

Electrophysiology

Direct methods of comparing the 'O' and 'B' systems to the RFamide system throughout development are not available. Instead, indirect electrophysiological methods were used to compare the development of the 'B' system in the tentacle bud to that of the RFamide system. The 'B' system is known to coordinate simultaneous contraction of the tentacles. In another hydromedusa, *Proboscidactyla flavicirrata* , when this system has synaptically connected the developing tentacle to the ONR, the tentacle will contract synchronously with the mature tentacles. Before this time, the developing tentacle contracts, due to endogenous contractions in the tentacle itself, but these contractions will not be synchronous with the other tentacles (Spencer, 1975). Suction electrode recordings were used to determine the stage at which the 'B' system is fully developed in the tentacle or at least connected to other 'B'neurons via the 'ONR'. The animals were anesthetized in a 1:1 mixture of sea water and 0.33M $MgCl_2$, and the suction electrodes were applied to two adjacent tentacles in each case: one adult tentacle; and one tentacle at one of stages 1 to 4 (n=18). Sea water was then slowly added, without disturbing the attachments of the suction electrodes, to dilute out the anesthetic. The contractions of the two tentacles slowly returned and were recorded. Extracellular recordings were made with flexible polyethylene suction electrodes with tip diameters approximately 100 μm in diameter. Electrical signals were amplified and displayed in a conventional manner (Spencer, 1978). The stage at which the 'B' system was fully formed, that is when contractions were synchronous, was

compared to histological findings of the RFamide neuronal system at each stage of development. Eighteen buds in different stages were compared to the adjacent mature tentacle. No indirect electrophysiological method was available to compare the development of the 'O' and RFamide neuronal systems in the bud. The swim motor neuron system was not examined because this system does not extend into the tentacle.

Results

Morphology of the developing tentacle

Polyorchis penicillatus is an anthomedusa of the family Polyorchidae. The adult medusa is characterized by a deep bell divided into four quadrants by radial canals with lateral diverticulae; a pronounced peduncle; sausage shaped gonads which are situated on the stomach; and a four cornered manubrium with frilly lips (Plate 2.1B-D). The asexual polyp form of this hydrozoan is still in question. Newly liberated medusae of *Polyorchis penicillatus* are 1mm in diameter and have four marginal (or perradial) tentacles, each with a red abaxial ocellus. The nearly spherical medusae lack gonads; the radial canals lack diverticulae; there is no peduncle, and the manubrium is simple (Plate 2.1A). There are also batteries of nematocysts, called cnidothylacies, on the exumbrellar surface of the bell (Plate 2.1A).

In this study, it was observed that the morphological development of each individual tentacle was very specific. Five developmental stages were identified (Plate 2.2; Figure 2.4). In Stage 0, no bud is visible on the basis of gross morphology. According to Skogsberg's tentacular series however, it is known that a bud will form in this location. Thus the ectoderm in this region is the presumptive epithelium for the developing bud. In Stage 1, the ectoderm thickens in the region of the future tentacle, forming a small bud at the bell margin (Plate 2.2A,B; Figure 2.4A). Within one day, in Stage 2, the endoderm from the ring canal outpockets into the ectodermal bud, to form the tentacle canal (Plate 2.2A,B; Figure 2.4B). Later the ocellus, or eyespot forms on the aboral surface of the tentacle bud. The ocellus is seen as a collection of red pigment, and the formation of the

Plate 2.1 The morphological characteristics of young and mature medusae from live specimens examined under a dissecting microscope. **A.** A newly budded medusa with four tentacles. Note the clusters of nematocysts (cnidothylacie) on the exumbrellar surface of the bell (arrowhead). There are six clusters per bell quadrant. There is no peduncle nor gonads, the radial canals lack diverticulae, and the manubrium lacks ornamentation. **B.** A more mature medusa with numerous tentacles. The cnidothylace are lost from the exumbrellar surface of the bell. Note the developing gonads on the peduncle (arrowhead). **C.** A close up of the diverticulae on a radial canal of an adult medusa. **D.** The manubrium of an adult medusa, note the development of 'lips'. Scale bar = 0.5 mm.

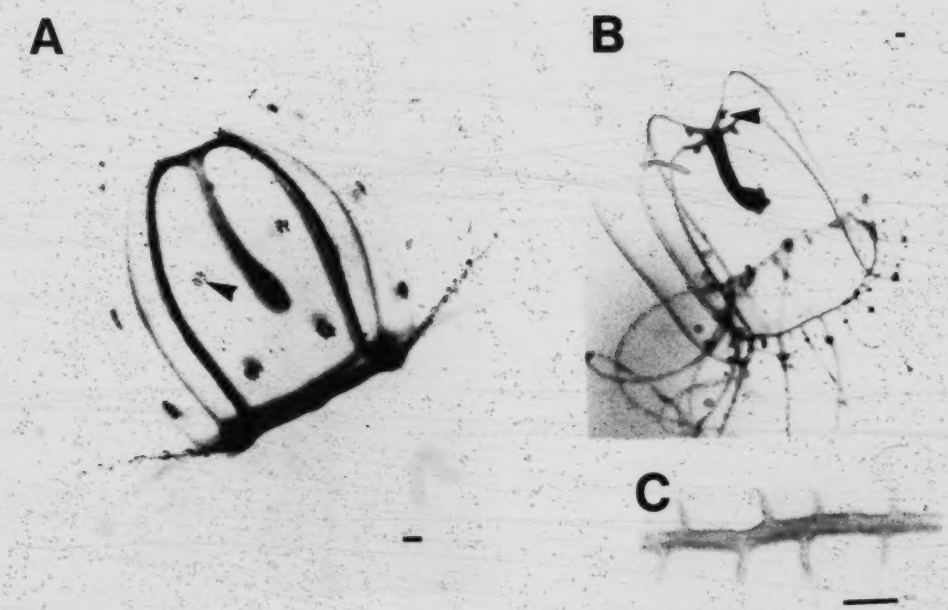

A
B
C
D

Plate 2.2 Stages of tentacle development in *Polyorchis penicillatus*. determined from live specimens examined under a dissecting microscope. **A.** Side view at the bell margin. The mature tentacle in the middle of the picture comes out towards the viewer. Stage 1: bud formation, ectodermal outpocketing only (LHS of **A** , arrowhead); Stage 2: bud formation, ectodermal and endodermal outpocketing (RHS of **A**, arrowhead). **B.** The bell margin viewed from above. Stage 1: bud formation, ectodermal outpocketing only (LHS of **B** , arrowhead); Stage 2: bud formation, ectodermal and endodermal outpocketing (RHS of **B**, arrowhead). **C.** Side view at the bell margin. The mature tentacles come out toward the viewer. Stage 3: development of the ocellus on the exumbrellar surface of the bud, which in this photo is the dark spot on the top of the bud indicated by the arrowhead. **D,E.** Side views at the bell margin. The mature tentacles come out toward the viewerStage 4: bud with tentacle rudiment elongation, at different lengths of development. **F.** Oblique view at the bell margin. Photograph to show branching of the endodermal canal in the tentacle (arrowhead). After the tentacle has reached full length, most of the further growth of the tentacle occurs proximal to this branching point. The stages are summarized in a line drawing (**Figure 2.4**)

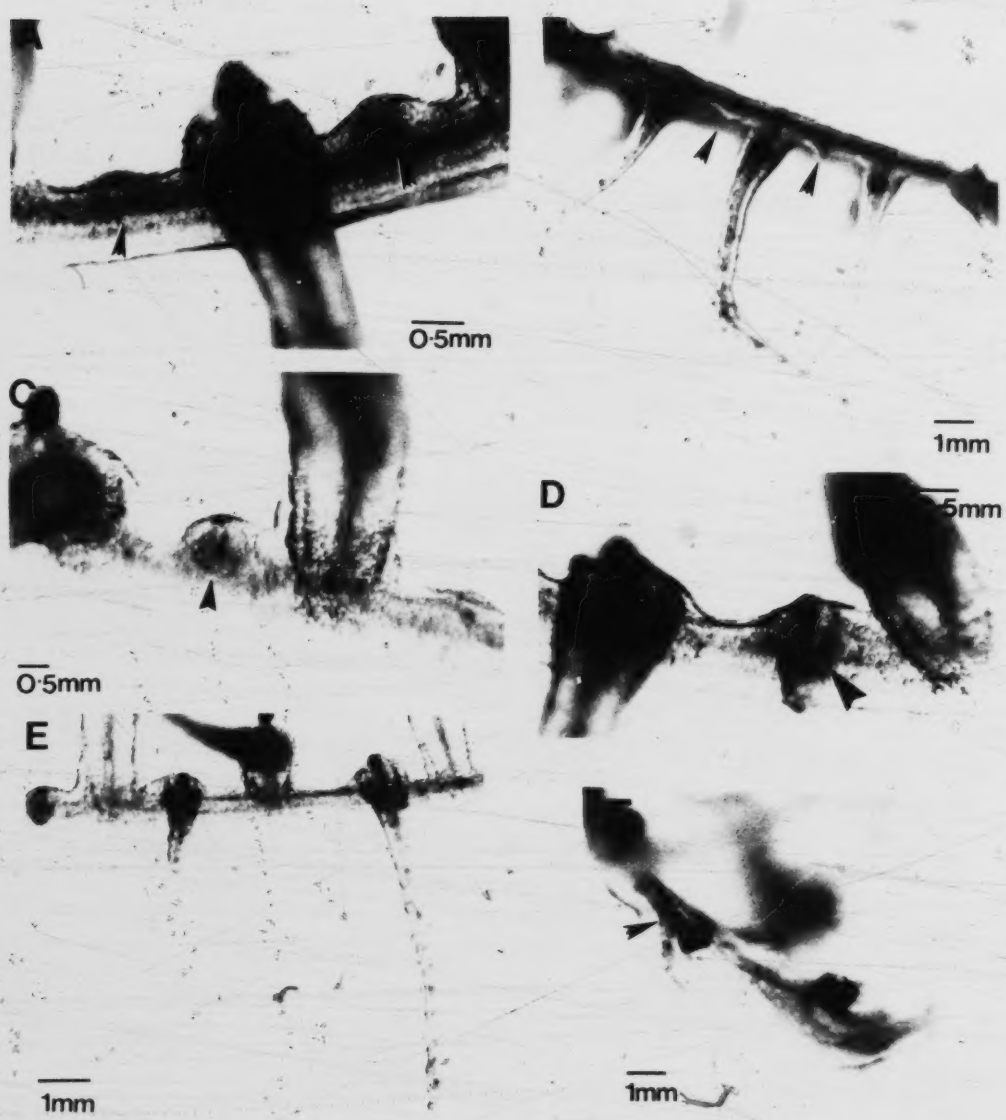
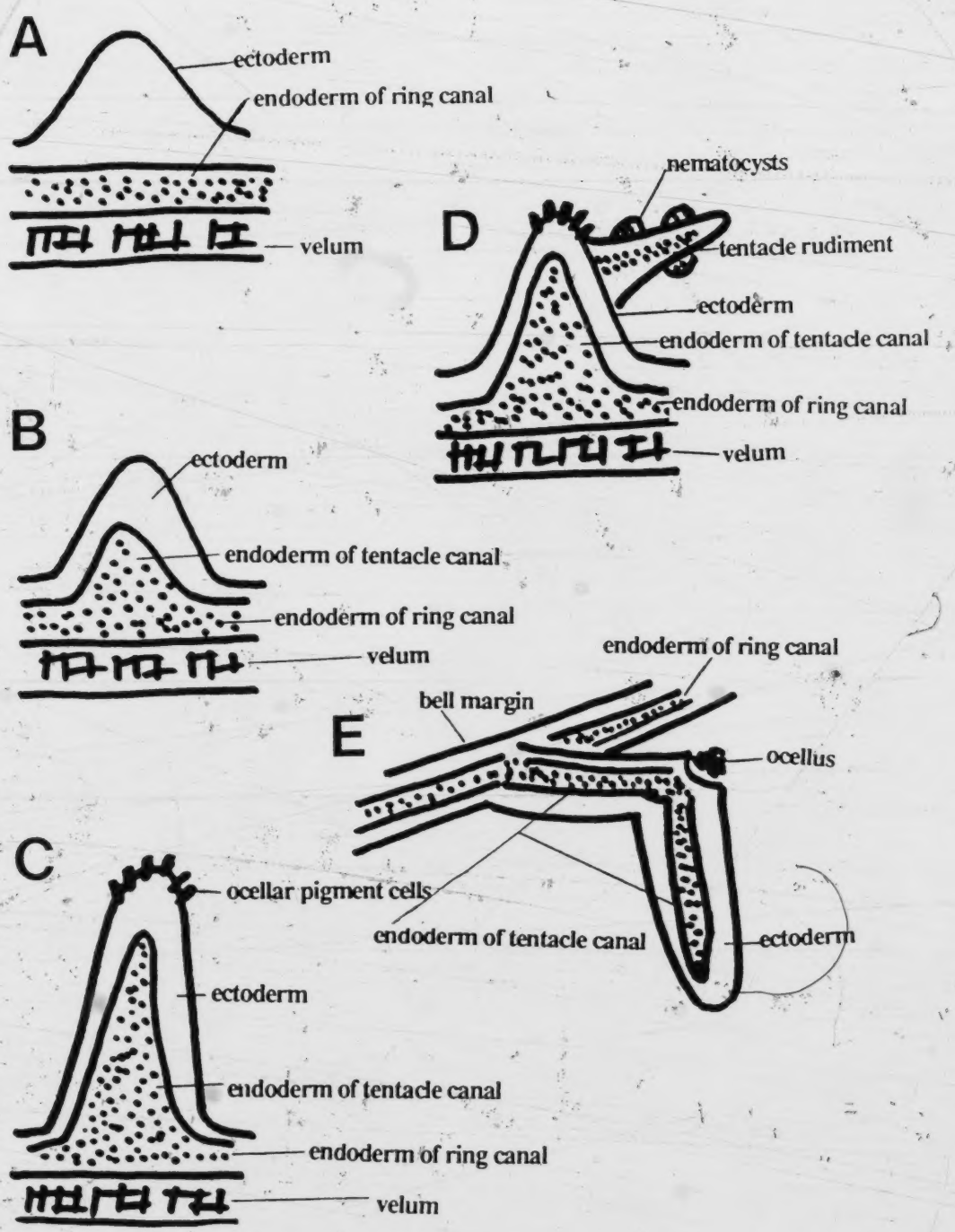

A
0·5mm
1mm
C
0·5mm
D
5mm
E
1mm
1mm

Figure 2.4 Line drawing showing the stages of tentacle development. **A.**Stage 1 bud, viewed from above. Exumbrellar surface is at top of the page, subumbrellar surface is towards the bottom of the page. Outpocketing of ectoderm only. **B.** Stage 2 bud viewed from above. Exumbrellar surface is at the top of the page. Outpocketing of ectoderm and endoderm. Endoderm begins to form the tentacle canal. **C.** Stage 3 bud viewed from above. Exumbrellar surface is at the the top of the page. Pigment cells have formed at the apex of the bud. **D.**Stage 4 bud viewed from above. Exumbrellar surface is at the top of the page. The tentacle rudiment has formed and is extending into the page and to the right. The endoderm of the tentacle canal branches at the ocellus and extends into the tentacle rudiment. **E.** Side view of a mature tentacle to show the growth in the subtentacular region, or the region of the tentacle between the bell margin and the ocellus. Also this diagram shows the branching of the endodermal tentacle canal at the ocellus, to extend into the tentacle rudiment.


A
ectoderm
endoderm of ring canal
velum
B
ectoderm
endoderm of tentacle canal
endoderm of ring canal
velum
C
ocellar pigment cells
ectoderm
endoderm of tentacle canal
endoderm of ring canal
velum
D
nematocysts
tentacle rudiment
ectoderm
endoderm of tentacle canal
endoderm of ring canal
velum
E
endoderm of ring canal
bell margin
ocellus
endoderm of tentacle canal
ectoderm

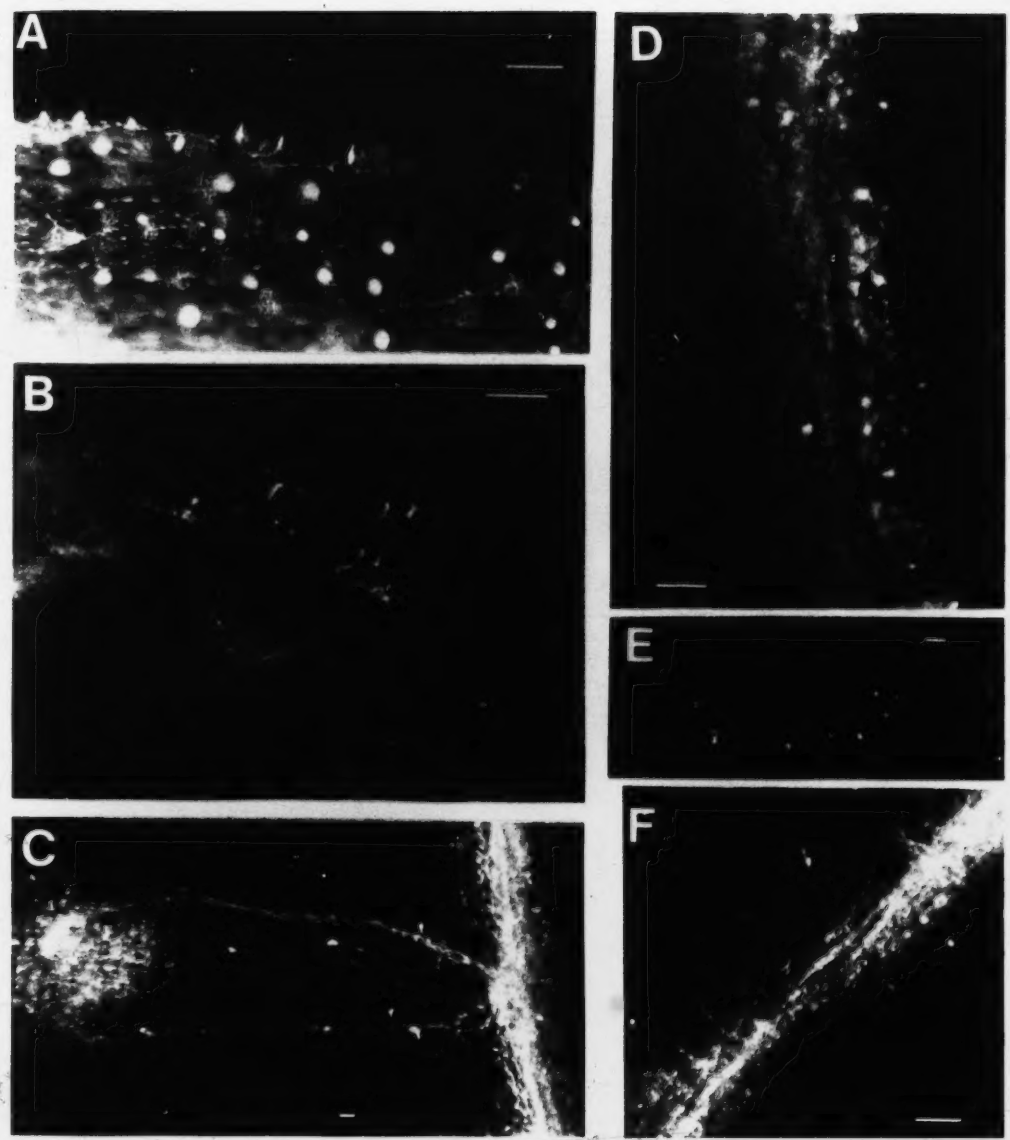
ocellus characterizes stage 3 (Plate 2.2C; Figure 2.4C). Once the ocellus has formed, the bud begins to elongate to form a tentacle rudiment (a stage 4 bud) (Plate 2.2D,E; Figure 2.4D). The endodermal tentacle canal branches at some point proximal to the ocellus (Plate 2.2F; Figure 2.4E). The tentacle rudiment appears to function physiologically as a mature tentacle. Over time, the tentacle rudiment grows to the length of other mature tentacles. As more tentacles are added to the bell margin, the older tentacles move up the bell, away from the margin, with all tentacles of a given 'whorl' located the same distance from the margin. The displacement of the tentacles laterally, up the bell results from tentacle growth in the region of the tentacle between the margin and the ocellus (Plate 2.2F; Figure 2.4E).

Development of the RFamide neurons with respect to tentacle development

RFamide immunoreactivity was observed in mature and developing tentacles of the anthomedusa *Polyorchis penicillatus*. Controls using preabsorbed anti-FMRF amide (100-500 µg FMRFamide/ml antisera) eliminated immunoreactivity. When anti-RFamide was omitted from the primary antibody solution no specific immunoreactivity was seen.

The RFamide system in the mature tentacle has been previously described (Grimmelikhuijzen and Spencer, 1984). The structure of the RFamide system in mature tentacles was examined again in this study to provide a baseline upon which to compare the staining patterns at the different developmental stages (Plate 2.3A-F), because the previous study used only frozen tissue, and in the current study fresh fixed whole-mount tissue was used. The RFamide neuronal system in the mature tentacle is comprised of aspects of both the peripheral diffuse plexus in the tentacle itself, and the 'central nervous system' in the bell margin which innervates the tentacle. In the tentacle distal to the ocellus, there is an immunoreactive nerve-net of small bi- and tri- polar neurons whose processes are beaded in appearance (Plate 2.3A). The perikarya are evenly spaced throughout the ectoderm, and they are often seen amongst a cluster of nematocytes, with an immunoreactive apical

Plate 2.3 RFamide-like immunoreactivity of the mature tentacle as shown with whole-mounts. **A**. The immunoreactive nerve net of the tentacle ectoderm. Perikarya are evenly spaced throughout the ectoderm, and are often seen in association with a cluster of cnidocytes which are identified in the photo as stipled areas of immunofluorescence which is due to autofluorescence. Scale bar=35μm. **B**. Immunoreactive cells of the ocellus lie at the periphery of the ocellus. These cells are elongate and often possess a slender immunoreactive process on their apical surface. Scale bar=60μm. **C**. The processes of the ocellar cells extend from the ocellus to the bell margin in two ocellar nerves. Note the lack of cell bodies in the ocellar nerves. The cell bodies which are seen on the extreme right of the photo, although they appear to be part of the ocellar nerves, actually lie in a plane above the ocellar nerves. Scale bar=15μm. **D**. Large immunoreactive cell bodies of the ONR. Tracts of immunoreactive processes are visible below the cell bodies. Scale bar=60μm. **E**. Cell bodies of the INR with immunoreactive tracts below. Scale bar=40μm. **F**. A region of the bell margin where the ocellar nerves can be seen entering the immunoreactive tracts of the nerve rings. Scale bar=60μm.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D
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F

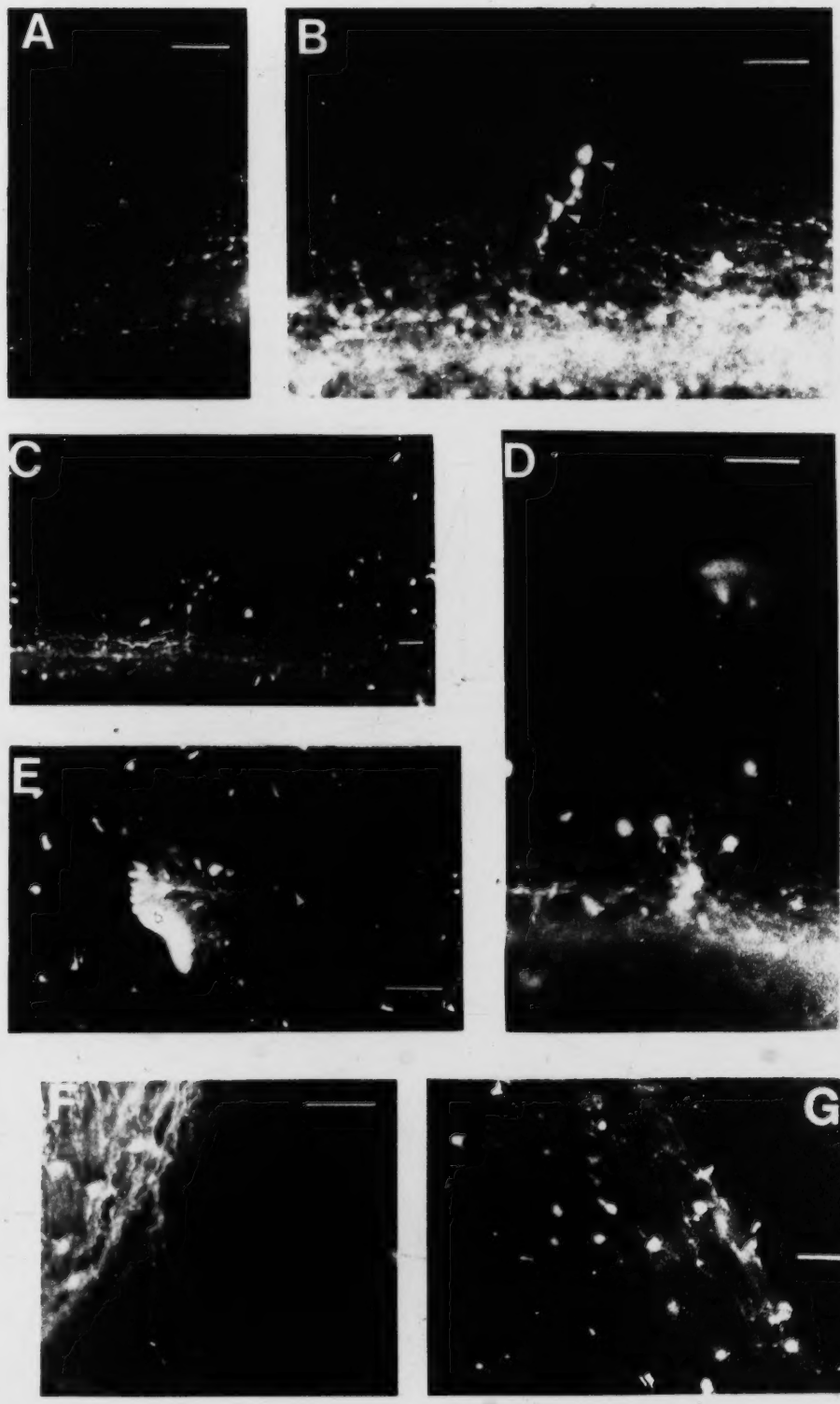
process extending from the cell body in the centre of the cluster of nematocytes (Plate 2.3A). Often an apical cilium is seen extending from the surface of these perikarya to the outer surface of the ectoderm. The immunoreactive nerve-net is also seen to approach smooth muscle of the ectoderm in the tentacle. Beading on the finer processes appear indicative of varicosities which may be synapses. Also, the finer processes parallel muscle processes. The ectodermal nerve-net is also present in the region of the tentacle between the ocellus and the velum, on the subumbrellar surface. The cells of this region appear to be largely bipolar, and the cell bodies appear to be arranged in vertical rows along the tentacle (Plate 2.3C). On the exumbrellar surface of the tentacle, proximal to the ocellus, no ectodermal nerve-net is present. This region is actually covered by the mesoglea of the bell. Some cells of the ocellus are also immunoreactive. These cells lie in a semicircle at the periphery of the ocellus (Plate 2.3B). The cell bodies are elongate (x=24μm, n=15), and often possess a slender immunoreactive process on their apical surface. The basal processes from these cells extend from the ocellus to the bell margin in two ocellar nerves on either side of the tentacle (Plate 2.3C). No cell bodies are seen in the ocellar nerves. In the intertentacular region of the ectoderm, at the bell margin, there is also a diffuse plexus of fine immunoreactive processes (Plate 2.3F). Some of these processes appear to connect the ocellar nerve of one tentacle to the ocellar nerve of the adjacent tentacle.

In the 'CNS', RFamide immunoreactivity is seen mainly in the largely sensory ONR, but is also seen in the largely motor INR. The immunoreactive cell bodies of both the ONR and the INR are uni- or bi-polar, elongate and bulbous with a diameter of 15 - 18μm (n=15, Plate 2.3D,E). The immunoreactive cell bodies are generally larger, and fewer in number in the INR than those of the ONR. Between these two bands of cell bodies lay several thick, wide, bands of immunoreactive tracts, which extend around the circumference of the bell (Plate 2.3D,F). No cell bodies are seen in this region. The ocellar nerves from the tentacle, as well as the processes from the ONR and the INR cells

can be seen entering this band of immunofluorescent tracts (Plate 2.3F). Although some cells in both the ONR and the INR are immunoreactive, the majority of cells of both nerve-rings, most notably the swim motor neurons of the INR, are not immunoreactive.

The neuronal subpopulations of the mature tentacle which show RFamide immunoreactivity are also seen in the different bud stages (Plate 2.4,A-G). The immunoreactive cells of the ONR and the INR are present throughout tentacle development. The ONR cells in the intertentacular region appear to be fewer in number than in regions adjacent to mature tentacles. The INR appears to contribute little, if any, direct innervation of the tentacle. In Stage 0, there is no unique immunoreactivity in the region where the bud will form, just the diffuse plexus of the intertentacular region (Plate 2.4A). When the ectodermal thickening is observed (Stage 1), there are typically one to two immunofluorescent cell bodies (n=26) in the bud, amidst the intertentacular network (Plate 2.4B). These cell bodies are approximately 15μm in length, unipolar, and have processes which extend to the immunofluorescent nerve tracts of the margin. The cell body is usually immunoreactive before the neurite shows staining. As the endodermal outpocketing develops (Stage 2), forming the early tentacle canal, more immunofluorescent cell bodies become visible. Typically, there are 12 - 16 cell bodies at this stage (n= 31), which are arranged at the tip of the bud in one or two cup-like arrangements around the endodermal canal (Plate 2.4C). All of the cells extend neurites to the nerve-ring. The bud continues to elongate to approximately 0.25 mm before the ocellus forms (stage 3, n=30). A few large cells (19μm) become immunoreactive at the bud apex, before the pigment cells of the ocellus are visible (Plate 2.4D). These cells are distinguishable from the cup-like arrangements of cell bodies, which are more proximal to the bell margin, and which remain at the base of the tentacle. The number of immunoreactive ocellar cells increases during the time that the ocellus is forming. As in the mature tentacle, the immunoreactive ocellar cells lie in a semi-circle around the periphery of the ocellus itself, and extend neurites in the

Plate 2.4 RFamide-like immunoreactivity of the tentacle bud stages. A. Diffuse plexus at the bell margin in the region of bud formation, prior to bud formation. Seen from the ONR side. Scale bar=50μm. B. Stage 1 bud with two immunoflourescent cell bodies extending neurites to the nerve rings. The two cell bodies are marked with arrowheads. The other sites of immunofluorescence are not cell bodies, but are varicosities along the length of the neurite. Seen from the ONR side. Scale bar=50μm. C. Cup-like arrangement of cell bodies around the endodermal canal in a Stage 2 bud. Seen from the ONR side. Scale bar=45μm. D. Large immunoreactive cells visible at the bud apex in a Stage 3 bud. Seen from the ONR side. Scale bar=60μm. E. Side view of a tentacle rudiment from the exumbrellar surface showing one of the ocellar nerves. The ocellar nerve is labelled with an arrowhead. Scale bar=60μm. F. Neurites connecting the ocellar nerve of a Stage 3 bud and the adjacent mature tentacle. Seen from the INR side. These neurites run parallel to the endoderm, and join to the immunoreactive cells at the bud apex. Scale bar=75μm. G. Ectodermal nerve net of the tentacle below the ocellus in a Stage 4 bud. Scale bar=45μm.


A
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D
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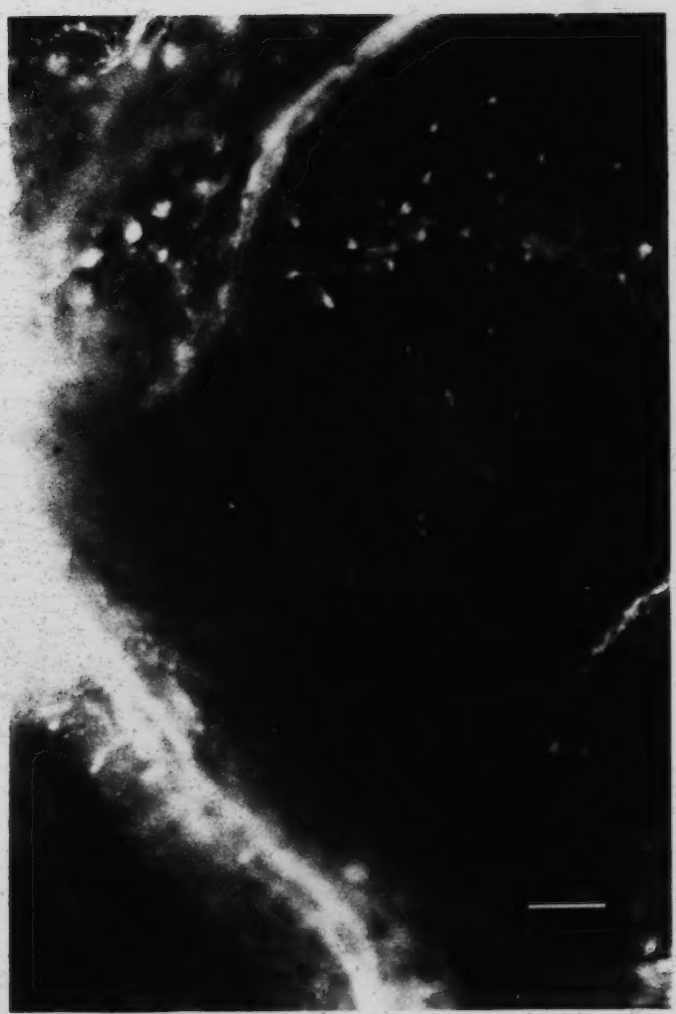
G

ocellar nerves to the bell margin (Plate 2.4E). When a bud is viewed from the INR side at the bell margin, there is also a diffuse plexus of fine immunoreactive processes (Plate 2.4F; Plate 2.5). Some of these processes run parallel to the outpocketing endoderm, and appear to form the ocellar nerves and connect with immunofluorescent cell bodies visible at the bud apex in Stage 3 buds (Plate 2.5). These neurites are also seen connecting the ocellar nerve of an adjacent mature tentacle to the ocellar nerve of the developing bud (Plate 2.4F; Plate 2.5) During, and after, ocellus formation, the endodermal canal branches at a point proximal to the ocellus and extends in a subumbrellar direction away from the bell margin. At this time of tentacle rudiment formation (Stage 4), the RFamide ectodermal nerve-net of bi- and tri- polar neurons become visible in the tentacle (Plate 2.4G). In the initial stages of rudiment development, the cell bodies of this immunoreactive network lie in longitudinal lines along the tentacle, both proximal and distal to the ocellus (Plate 2.4G, n=34). The tentacle rudiment continues to elongate until a young tentacle is formed. These young tentacles appear to function physiologically, at least with respect to their responses to light, in a similar manner to mature tentacles.

The development of other identified neurons in relation to tentacle development

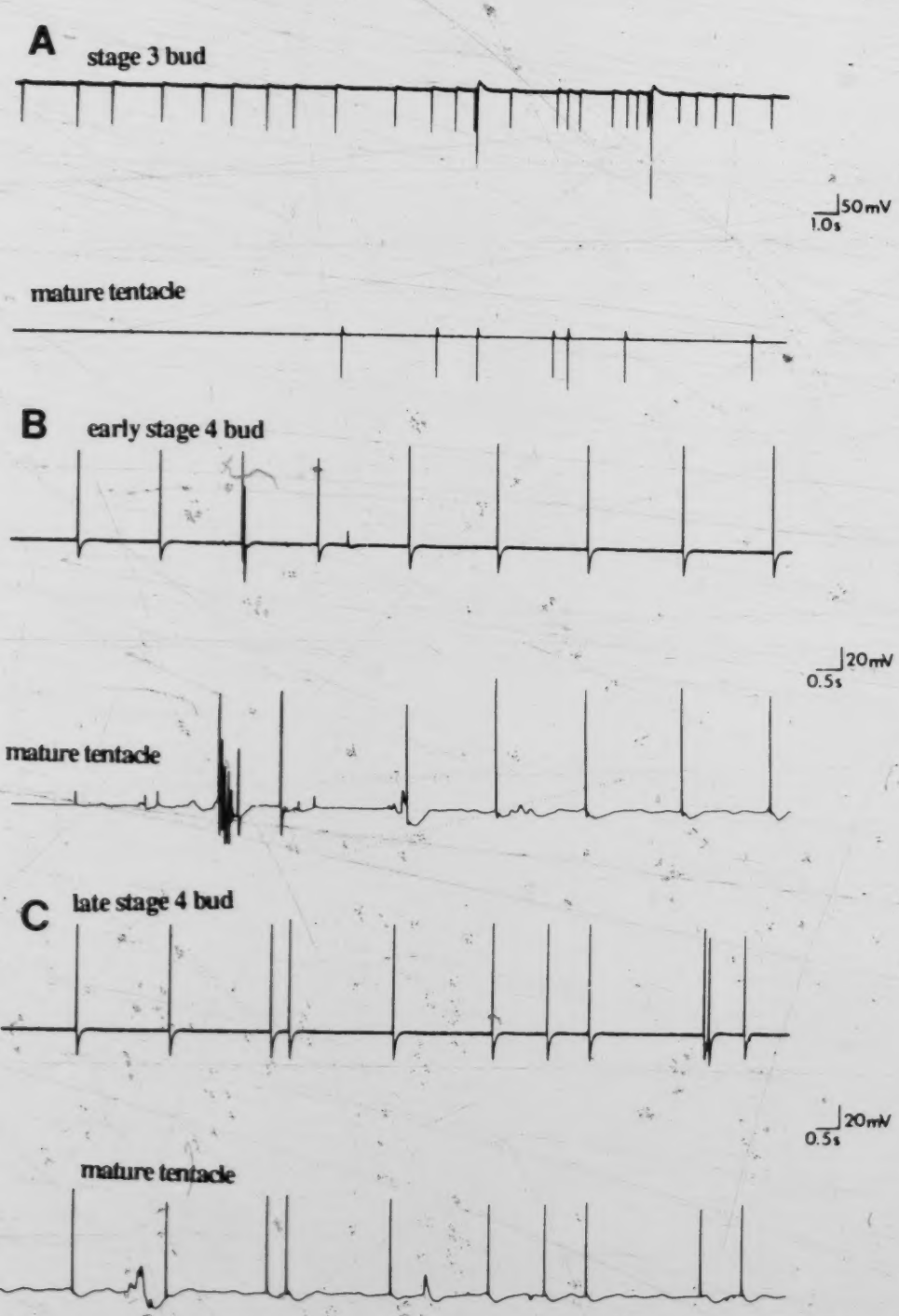
Suction electrode recording experiments which indirectly compared the development of the 'B' system to tentacle morphology, demonstrated that the 'B' system became connected to the ONR in the later stages of bud development (n=18). The 'B' system neurons were identified by their spiking activity which correlated with tentacle contractions. The 'B' system is not fully developed in Stage 1 and Stage 2 buds, where no spontaneous contractions of the tentacle bud were visible, either by visual observation, or as determined by recording with suction electrodes. Consequently, there were no synchronous contractions in these early bud stages, although, if the electrode is attached with strong suction to the bud, the 'B' system can be recorded from the nerve-ring in this

Plate 2.5 The RFamide-like immunoreactivity of the developing tentacle at stage 3, viewed from the INR side. A very early Stage 3 bud viewed from the INR side. Here it can be seen that the ocellar nerves connect the bud to the adjacent tentacles, and that the ocellar nerves are connected to the immunoreactive neurons visible at the bud apex. Scale bar=80μm.



region. Stage 3 buds (ocellus only) were found to contract spontaneously but asynchronously, even during bursts of contractions (Figure 2.5a). Early Stage 4 buds, however, were found to alternate between synchronous and asynchronous contractions (Figure 2.5b). When the tentacle rudiment had begun to elongate further than about 3 mm, it was found that the contractions of the tentacle rudiment were synchronous with the mature tentacles of the medusa (Figure 2.5c). No method was found to compare the development of the 'O' system with tentacle development.

Figure 2.5 Extracellular recordings of the 'B' system in tentacle buds of different stages, together with a mature tentacle. In all three examples, the mature tentacle recording is the lower trace. **A**. Recording of spontaneous 'B' system activity from an early Stage 3 bud. Note the consistently asynchronous spontaneous contractions. The tentacle bud contractions are asynchronous even through bursts of activity. **B**. Recording from an early Stage 4 bud. The contractions alternate between being asynchronous and synchronous with the mature tentacle. This type of activity is seen in both late Stage 3 and early Stage 4 buds. **C**. A recording from a late Stage 4 bud and a mature tentacle. The contractions are consistently synchronous, although a given tentacle is still capable of making individual contractions (see C, bottom trace RHS).


A
stage 3 bud
50mV
1.0s
mature tentacle
B
early stage 4 bud
20mV
0.5s
mature tentacle
C
late stage 4 bud
20mV
0.5s
mature tentacle

Discussion

In the mature tentacle of *Polyorchis*, it seems unlikely that there is only one system of RFamide immunoreactive neurons. On the basis of histology alone there are at least four potentially different neuronal systems immunoreactive to anti-RFamide which may or may not be interconnected. These four systems are: the immunoreactive neurons of the ectodermal nerve-net in the tentacle; the immunoreactive ocellar cells; the immunoreactive neurons of the INR; and the immunoreactive neurons of the ONR.

The ectodermal nerve-net of RFamide-positive neurons in the tentacles are associated in part with nematocytes, and may therefore be part of a system which modulates sensitivity or stimulus threshold of nematocytes. Nematocyte discharge does not seem to be under direct nervous control (Pantin, 1942a,b; Jones, 1947; Burnett *et al.*, 1960; Lentz, 1966; Picken and Skaer, 1966), however synapses are known to be present between neurons and nematocytes in hydromedusae, with dense-cored vesicles in a size range consistent with vesicles containing neuropeptides (Westfall, 1970). In *Hydra* and some anemones, more recent evidence suggests that the animal itself is able to control the threshold for nematocyst discharge through a mechanism (perhaps neuronal) which is able to suppress nematocyst discharge in satiated animals (Sandberg *et al.*, 1971; Sandberg, 1972; Mariscal, 1973; Smith *et al.*, 1974), or increase discharge after handling animals (Conklin and Mariscal, 1976). Also, the immunoreactive nerve-net of the tentacle contains processes which approach smooth muscle cells of the tentacle ectoderm.

The ocellus is a photoreceptive organ and the RFamide immunoreactive ocellar cells may have a sensory function. In the adult tentacle the cell bodies are arranged in a semicircle around the pigment cells. The immunoreactive ocellar cells are similar in

structure and location to the second-order neurons of the ocellus described in hydromedusae by Toh *et al.* (1979) and others (Singla and Weber, 1982a,b). In some second order neurons a distal prolongation of the nerve cell reaches the exterior surface of the ocellar protuberance and there projects a cilium. The RFamide cells of the ocellus are also seen to extend to the exterior with a distal protuberance. Like RFamide cells, each second order neuron has a single axon which leaves the ocellus as part of a bundle of the ocellar nerve (Toh *et al* ., 1979). *Sarsia tubulosa*, however, lacks second order neurons, and yet still shows RFamide immunoreactivity in the ocellar nerve, although this immunoreactivity is obscured as the ocellar nerve enters the tentacle bulb (Singla and Weber, 1982b). RFamide-positive cells of the ocellus might have a role in modulating the photic responses of medusae. Studies in teleost retina have shown that FMRFamide is found in centrifugal fibres originating from the olfactory system, and synapsing onto dopaminergic interplexiform cells (Stell *et al.*, 1984; Zucker and Dowling, 1987). It is thought that the FMRFamide-positive cells in the teleost retina may modulate light responsiveness and receptive field size of horizontal cells in the teleost outer plexiform layer (Zucker and Dowling, 1987). Immunoreactive cells of the ocellar nerve proper may serve in some capacity in the control of smooth muscle, as processes of the ocellar nerve are clearly seen connecting with the tentacle nerve-net, and connecting with the adjacent tentacle.

The function of the immunoreactive cells of the INR and the ONR are unknown. From present observations however, it is clear that neuronal processes do cross the mesoglea between the two nerve-rings. As well, it seems likely from present observations, that the cells of the INR contribute little to the innervation of the tentacle proper, unless it is through another system of neurons.

Immature tentacle buds were examined as it seemed likely that the RFamide immunoreactive systems might show stages of development which would shed light on the

function of these systems in the mature tentacle. The four systems described in the adult were also seen in different stages in the immature tentacle buds. The cells of the INR and the ONR are present throughout tentacle development. The number of immunoreactive cells in the two nerve-rings appears to increase during the development of the tentacle. The ocellar cells and ocellar nerves become visible in stage 3 buds. At first, only one or two of these large cells are present at the tip of the bud. As the ocellus forms however, the number of RFamide-positive cells in the bud tip increases until the number and distribution of these cells is is similar to the mature tentacle. The ectodermal nerve-net of the tentacle proper is visible in late stage 3 and stage 4 buds. In addition to these four systems there is another population of immunoreactive neurons present in stage 1 and stage 2 buds, which is not present in the mature tentacle. These neurons form a characteristic 'cup-like' arrangement of cells at the base of the Stage 2 bud. This system of neurons is not accounted for in the immunoreactive systems described in the mature tentacle.

These immunoreactive neurons present in Stage 1 and Stage 2 buds form a distinct population of neurons which are present only during tentacle development. Similarly, in the planulae larvae of *Halocordyle* the RFamide phenotype is expressed at a critical stage in development of planular attachment and metamorphosis (Martin, 1988). The developmental correlation between the presence of RFamide-like peptides in the nervous system of the planulae and metamorphosis of the larvae suggests that the peptides may play a crucial role in the development of these larvae, or that the peptides may be important in metamorphosis itself (Martin, 1988). A similar situation may exist for tentacle development in *Polyorchis* and the appearance of this subset of immunoreactive neurons in Stage 1 and 2 buds.The fate of this population of immunoreactive neurons which are present only in early bud development, be it cell death, migration, or a change in phenotype, is not known. The function of this neuronal subpopulation is not known, but their presence in early bud stages suggests that these neurons play some role in the early

development of the tentacle. There may be, as in *Hydra*, a threshold number of neurons which are required to establish conditions which allow for the first tentacular buds to develop (Bursztajn and Davis, 1974). In *Hydra* as soon as neurons accumulate in the tentacular primordia, small outpushings of the tentacles occur, however , before this time tentacular growth is not observed (Burstajn and Davis, 1974). Similarly, Schaller (1981) found that neurons accumulate locally just prior to budding in *Hydra*, and suggested that a surge of neuron differentiation might be responsible for bud initiation. Berking (1980) found that the accumulation of neurons prior to budding in *Hydra* was the velocity-determining step in bud development. Also, in *Phialidium gregarium* and in *Mitrocomella polydiademata* neurosensory cells seem to play and important role in metamorphosis of the planula larvae of these species (Thomas *et al.*, 1987; Martin *et al.*, 1983). Unfortunately, no one has determined in any of these examples whether these neurons are RFamide-positive. Alternately, the neuronal subpopulation in the *Polyorchis* tentacle bud might provide a pathway by which the other neurons can enter the bud. The medusa *Aglantha digitale*, has 'star cells' in its tentacles which are also immunoreactive to RFamide, and which are thought to be guidepost neurons of some sort, for other neurons entering the tentacle (Mackie, pers. comm.). In the developing wings of the moth *Manduca sexta*, pathways from the appendages to the CNS are initially laid out by neuronal pioneering fibres (Nardi, 1983).

Evidence of morphogens in cnidarians is extensive, and includes a variety of compounds. For example, homarine (N-methyl picolinic acid) is an extract of cnidarian tissue homogenate which is contained within the tissues of both *Hydractinia* and *Hydra*. Homarine retards metamorphosis in *Hydractinia*, and influences the proportioning of the future polyp's pattern formation in both *Hydractinia* and *Hydra* (Berking, 1986). Another morphogen is the neuropeptide Head Activator (pGlu-Pro-Gly-Gly- Ser-Lys-Val-Ile-Leu-Phe) which is found chiefly in neurosecretory granules of nerve cells of *Hydra* (Schaller

and Gierer, 1973). Head Activator activates head formation in *Hydra* during morphogenesis by stimulating multipotent precursor cells (interstitial cells) to differentiate into nerve cells, and also by inducing cells capable of proliferation to divide (Schaller, 1973; Grimmelikhuijzen and Schaller, 1979; Schaller and Bodenmüller, 1981; David and Holstein, 1985). In *Hydractinia* , a factor, PAF (proportion activating factor) causes an increase in head formation, and an increase in the number of multipolar (ganglion type) RFamide-positive neurons (Plickert, *et al.*, 1987). The presence of a variety of amines such as dopamine, norepinephrine, and serotonin, have been demonstrated in *Hydra.* (Hanai and Kitajima, 1984; Venturini *et al.*, 1984). Since these amines have not been shown to be active in the animal's behaviour and are not involved in control of adenylate cyclase activity (Venturini *et al.*, 1984), it is thought that these amines may act as morphogenic factors like Head Activator. The possibility, however, that these substances are involved in neurotransmission is not excluded (Hanai and Kitajima, 1984; Venturini *et al.*, 1984). Catecholamines have also been localized in the planula larvae of *Halocordyle*, and the exogenous application of the catecholamine, norepinephrine to planulae of *Halocordyle* results in premature rapid metamorphosis of the larvae, suggesting a possible morphogenic role of catecholamines in metamorphosis which could be indirect by initiating release of morphogenic neuropeptides (Kolberg and Martin, 1988). Such a role for the RFamides in *Polyorchis* might also be envisioned.

Recently, RFamide has been localized immunocytochemically with immunogold labelling to dense-cored vesicles in the outer nerve-ring of *Polyorchis* (Singla, unpublished data). Vesicles at synapses were not labelled. Although only the outer and inner nerve-rings have been examined, the restriction of label to non-synaptic vesicles, suggests a neuromodulatory, neurohormonal, or morphogenic role for the RFamides rather than a role as a neurotransmitter.

Further experiments to characterize these RFamide neurons are needed. If one could recognize the immunoreactive neuronal subpopulation present in stage 1 and Stage 2 buds, fill these cells with Lucifer Yellow, and selectively ablate the neurons with a laser, then one could examine how the bud would develop in the absence of these neurons. Another way of examining this problem might be through passive immunization of the medusa with anti-RFamide. Presumably, the antibodies in the extracellular fluid would bind any released RFamide peptide. Two problems coincident with this technique are that penetration into the extracellular area might be difficult, and the clearance of the foreign antibodies might be very rapid, rendering the technique ineffective.

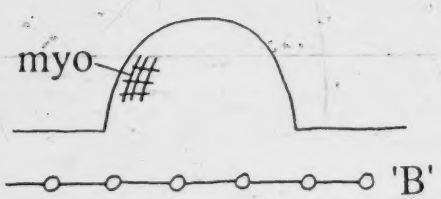
One problem in examining the neurons of the bud stages is that no method has been found to visualize other neurons present in the bud (Appendix I). Previous studies (Grimmelikhuijzen and Spencer, 1984; Mackie *et al.*, 1985) had provisionally matched the RFamide immunoreactive neuronal system with the physiologically identified 'O' and 'B' systems. Preliminary double-labelling techniques, however, which allowed simultaneous visualization of iontophoresed dye in the 'B' (or the 'O') system, and the immunocytochemical staining of the RFamide system with a rhodamine label, have shown that the 'O' and 'B' neuronal subpopulations are distinct from the RFamide system (Spencer, pers. comm.).

Indirect evidence in the present study seems to show that the 'B' system develops in the tentacle at a much later date than the RFamide systems in the tentacle, although it is recognized that it is possible that the 'B' system phenotype could be expressed without being active. In the mature tentacle, the 'B' system is known to control synchronous tentacle contraction (Spencer and Arkett, 1984). In early bud stages, (1 and 2), no RFamide nerve-net is present, and there are no spontaneous contractions of the bud, or synchronous contractions of the bud with the other tentacles, and the 'B' system can be recorded from the ONR. In stage 3 buds, the beginnings of the RFamide ectodermal net

are seen. Stage 3 buds contract spontaneously, but not synchronously with other tentacles. These endogenous contractions are perhaps due to the neuromuscular connection of the RFamide neurons to the smooth muscle cells in the individual tentacle. At this time the 'B' system is not recorded in the bud. Early stage 4 buds contract synchronously with the mature tentacles. As the tentacle rudiment forms the RFamide ectodermal net becomes more extensive and the 'B' system can be recorded from the tentacle rudiment. One possible explanation is that initially the RFamide system causes newly developing tentacle to contract spontaneously and asynchronously, then as the'B' system develops and innervates the tentacle, it becomes synaptically connected to the RFamide nerve-net in the tentacle to produce synchronous tentacle contraction. The connections and arrangements of these components are summarized in Figure 2.6. One problem with this scenario is that the preliminary immunocytochemical data suggests that RFamide is not located at synaptic vesicles (Singla, unpublished data). However, Singla has not yet looked at neuromuscular synapses, so it is possible that RFamide synapses could be present in the ectodermal nerve-net of the RFamide system, even if such synapses are not seen in the neuro-neuronal immunoreactive networks of the INR and the ONR. Koizumi *et al.* (1989) found that immunogold labelling of RFamide in *Hydra* labelled dense-cored vesicles in axon terminals at epitheliomuscular cells, suggesting neuromuscular transmission in *Hydra* utilizes RFamide peptides although again, no typical synaptic loci were observed. Another possibility is that RFamide might be released at random sites, in a non-phasic way, and act to cause sensitivity to 'B' system spikes, allowing synchronicity of tentacle contraction as in *Renilla*, where FMRFamide does not have a direct effect on effectors but causes an increase in excitability of the nerve-net (Anctil, 1987). It is recognized, however, that there are a number of methods by which synchronicity of tentacle contraction could be obtained besides through the RFamides increasing sensitivity. For example, local 'B' neurons in the tentacle might connect with marginal 'B' neurons at the time the RFamide

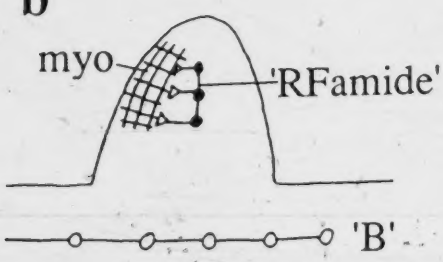
Figure 2.6 Schematic diagram illustrating a possible connection of the 'B' system to the RFamide system during tentacle development. **A.** Stage 1 or Stage 2 bud. **B.** Early Stage 4 bud. **C.** Later Stage 4 bud. myo, myoepithelial cells; 'B', 'B' system neurons; RFamide, RFamide ectodermal nerve net.

a



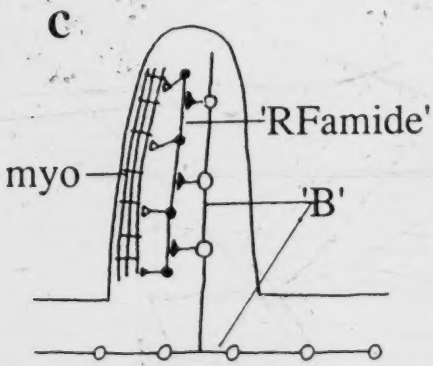
1. No RFamide ectodermal net
2. No spontaneous contractions
3. No synchronous contractions
4. 'B' system recorded from ONR

b



1. RFamide ectodermal net present
2. Spontaneous contractions
3. No synchronous contractions
4. No 'B' system recorded in tentacle

c



1. 'B' system innervates tentacle
2. RFamide system connected to 'B' system
3. Spontaneous contractions
4. Synchronous contractions with mature tentacles
5. 'B' system recorded in the developing tentacle

neurons proliferate. Similar electrophysiologic evidence is not available for comparing the RFamide system to the 'O' system, or any other systems which may be present in the tentacles. Monoclonal antibodies to other neuronal subpopulations would have been useful in comparing the different subpopulations of neurons in the developing tentacle to the RFamide-positive neurons, but all efforts to develop monoclonal antibodies that are specific for neurons have been unsuccessful to date (Appendix I).

Intercellular communication is important for organizing the spatial and temporal pattern of cellular differentiation. Peptides in general are extremely well suited to fulfil a fine-tuning role of regulation between cells in a simple nervous system because peptidergic transmission can provide a more generalized method of signalling, where peptides may be exocytosed at any part of the plasma membrane as seen by Singla's (unpublished data) evidence of RFamide immunoreactive dense-cored vesicles found throughout the neurons. As well, peptides can have different effects on a given target cell such that one peptide, for example, an RFamide-like peptide, might have multiple functions within different subpopulations of a nervous system simply due to the type of cells upon which the peptide has its effect. The peptide might function as a neurotransmitter in one population, a neuromodulator in other systems, and as a hormone or morphogen in yet another, based on the receptors of the target cells. Or indeed, a family of RFamide peptides which differ from each other by only a few amino acids might provide multiple functions within different subsets of neurons. Target-cell sensitivity to the neuropeptide or the type of neuropeptide synthesized might change over time as well, allowing a further diversification of function, and different pattern formations. This general nature of neuropeptides may be more appropriate in simple nervous systems such as in the cnidarians, which lack a high degree of specialization. Multifunctional neurons and target cells could employ 'systems' cells acting in coordination as described above to perform those functions necessary for the

maintenance and growth of the organism, which in other more sophisticated animals would be performed by different groups of more specialized cells or organs.

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III. Growth and regeneration of tentacles of *Polyorchis penicillatus* in a culture system.

Introduction

The relationship of neurons to regenerative capacity of a body part is founded in the work of Singer (1952) who found that a threshold number of nerves is required to exert a neurotrophic effect on limb regeneration in the amphibian *Triturus viridescens*, without which the limb will not regenerate. The most critical time of nerve dependency in regeneration is the period of initial outgrowth of the limb. Once initial outgrowth is underway, regeneration of the limb can proceed without innervation (Schotte and Butler, 1944). Studies of other phyla, both vertebrate and invertebrate, have revealed this phenomenon of nerve-dependent regeneration to be widespread, although the majority of such work continues to use amphibian systems. The nervous system has been implicated as an essential component for regeneration in planarians (Child, 1920) and arthropods (Needham, 1952). Neurons are indispensable for regeneration in annelids where they penetrate the wound ectoderm and produce neurosecretory hormones (Herlant-Meewis, 1964). Neurons also play an important role in the regeneration of nemerteans (Tucker, 1959). In mammals, nerves exert an influence on mitosis, differentiation and morphogenesis of taste buds, and are required for the maintenance of functioning phenotypes in muscle (Zalewski, 1969; 1970; Wessells, 1977).

Most studies of regeneration in the Hydrozoa have concentrated on regeneration in hydroid polyps. These studies have revealed much about the capabilities of these animals to regenerate, and the role that neurons might play in such processes. In hydroids, it has been suggested that control of growth and regeneration is mediated by neurosecretory substances produced by nerve cells (Lentz and Barnett, 1963; Burnett *et al.*, 1964; Lentz,

1965a,b; Lesh and Burnett, 1966; Schaller, 1973; Bursztajn and Davis, 1974; Yaross *et al.*, 1985). Burnett and Diehl (1965) indicated that there is an increase in 'neurosecretory droplets' in neurons for a period of 4 hours following a cut. The density of nerve cells has been correlated with the rate of bud production in *Hydra* and related to trophic effects due to release of neurohormones (Shostak, 1974; Berking, 1980). In *Hydra attenuata*, regenerating animals produce more nerve cells at the regenerating apical tip than do normal animals (Bode *et al*,, 1973). Although difficult to study, due to the diffuse nature of the nervous system, it appears that neurons may also play a role in regeneration of medusae.

In the Hydrozoa, neurons develop from interstitial cells. Interstitial cells are multipotent stem cells, of which, approximately 40% produce nerve cells and several types of cnidocytes (David, 1980; Heimfeld and Bode, 1984a; 1984b). Chemical elimination of interstitial cells eliminates the neurons which would have formed subsequently as the interstitial cells are the neuronal precursors. Diehl and Burnett (1964) showed by chemical elimination of interstitial cells using hydroxyurea that interstitial cells are not necessary for regeneration events in *Hydra*, but that regeneration will not occur if neurons are not present in the regenerating tissue. In later studies of *Hydra* and other hydroids rendered 'interstitial cell free', it was shown that some neuronal types could regenerate despite the lack of interstitial cells, while other neurons could not, suggesting that some neurons are able to form from other types of cells presumably through transdifferentiation (Yaross *et al.*, 1985; Bode *et al.*, 1986; Koizumi and Bode, 1986; Martin, 1988). RFamide-positive neurons from medusae have been shown to transdifferentiate from muscle cells *in vitro* (Schmid and Alder, 1984). In *Hydra* , there is evidence for *in vivo* transdifferentiation of an RFamide-negative neuron into an RFamide-positive neuron which is position-dependent (Bode *et al* ., 1986; Koizumi and Bode, 1986).

Much less is known about the regenerative capacities of hydrozoan medusae, and the role neurons may play in their regeneration. Typically, the regenerative potential of

medusae is less than that of polyps (Schmid and Tardent, 1971). Few studies have examined the regenerative potential of medusae, and those studies which have examined medusa regeneration have examined the regenerative capacity of the bell itself (Schmid and Tardent, 1971; Schmid, 1974), not that of the bell margin or of the tentacles, where the sensory and motor components of the nervous system are concentrated. The regenerative capacity of RFamide-positive neurons themselves, or their capacity to induce regeneration, has not been examined.

In *Polyorchis penicillatus*, the regenerative potential of the bell margin, the tentacles, or of its neurons is unknown. The present study examines the regenerative potential of tentacles in *Polyorchis* to determine the extent to which regeneration occurs in *Polyorchis*. The development of regenerating tentacles is compared to tentacles formed during ontogeny. One system of neurons which innervate the tentacles, the RFamide immunoreactive neuronal system, is examined during regeneration, and the regeneration of this neuronal system is compared to the formation of this neuronal system during ontogeny (See Chapter 2). Lastly the regenerative and ontogenetic potential of tentacles in different developmental stages is examined in the absence of neuronal precursors (interstitial cells), to determine whether neurons play a role in growth and regeneration, and if so, at what stage of development or regeneration they are important.

Methods and Materials

Tentacle development during ontogeny and regeneration

Specimens of *Polyorchis penicillatus* (bell diameter 0.2 - 1 cm) were collected by plankton tow from Bamfield Inlet, Bamfield, B.C. The animals were examined, and the number of tentacles, and tentacle buds were determined, and drawn (n=79). The animals were maintained in a standing sea water culture at 10°C. The water was changed twice daily, and the animals were fed once daily with live copepods, *Tigriopus californicus*, which were collected intertidally. Although this food source is not the natural diet of *Polyorchis*, the medusae were seen to ingest, and digest, the copepods. It was thus assumed that the medusae recieved nourishment from this food source, although weight changes were not measured. The animals were examined at least daily, and any tentacle growth was noted. In addition, the animals were checked for a normal response to shadows, namely several swimming contractions. A normal response was considered to be an indication of a healthy animal. In addition to these animals maintained in culture, a further 190 individuals of varying sizes were collected. The bell diameter of these animals was measured as an indicator of size, and it was compared to the number of tentacles the medusae had. The results were plotted as a bar graph.

To examine the regenerative potential of tentacles, a series of extirpation experiments were performed on medusae with tentacles in different developmental stages (described in Table 3.1) The developmental stages of tentacles are described in Chapter 2. The extent of tentacle regeneration at each stage in animals of different ages (as determined by tentacle number) was examined (n=6 per developmental stage in each age of animal,

Table 3.1 Summary of Extirpation Experiments

1. Stage 1 bud ablated.
2. Stage 2 bud ablated.
3. Stage 3 bud ablated.
4. Stage 4 bud ablated.
5. Mature tentacle ablated.
6. Ocellus removed from stage 4 bud.
7. Ocellus removed from mature tentacle.

except where otherwise noted). Buds or tentacles were removed from animals by applying suction to the bud for about 30 minutes with a plastic suction electrode (dia.=1mm) attached to a 10mL syringe. After extirpation, some animals were fixed immediately to check the effectiveness of the extirpation, to determine that the bud, and all of the RFamide neurons, were removed with extirpation (n=2 per stage). The criteria for a successful extirpation were that all morphological signs of the bud were removed, and that when stained with anti-RFamide, the immunoreactive profiles which would have normally been seen at that bud stage, were no longer present. In the remaining animals (n=4 per stage, per age), the long tentacles on either side of the extirpated region were trimmed after extirpation to mark the extirpation site. These long tentacles regrew and it was necessary to trim them every few days. After a few hours recovery in natural sea water, with gentamycin (50 mg/l) added as protection against bacterial infection, the animals were maintained in the standing sea water cultures described above. The animals were examined at least daily, and specific changes in the gross morphology of the extirpated bud were noted. When these morphological changes occurred, the animals were photographed, and some of the animals were fixed and stained for whole mount immunocytochemistry of anti-RFamide, as described in Chapter 2. The number of regenerated buds in each stage stained with anti-RFamide was as follows: Stage 1, n=10; Stage 2, n=10; Stage 3, n=10; Stage 4, n=8. The animals which were fixed were replaced in culture by animals which had had the same extirpation performed on them so that a total of 4 animals, per stage, per age were followed completely through tentacle formation. Changes in tentacle morphology during regeneration was compared to the normal stages of tentacle development, and the pattern of RFamide immunoreactivity for each stage, described in Chapter 2. The time spent in different developmental stages during regeneration was compared to the time spent in each stage during ontogeny as determined in the culture system described above. In addition to regeneration in the bud stages, regeneration of ocelli and mature tentacles was also

examined. The experiments were performed as described above, and are summarized in Table 3.1.

Tentacle development and regeneration in the absence of interstital cells

Hydroxyurea (Sigma) treatment, which causes selective depletion of interstitial cells and their progeny (Bode *et al.*, 1976) was used to determine whether the absence of neurons, derived from interstitial cells, affected ontogenetic growth or regeneration of the tentacle. It was first necessary to determine the time required in hydroxyurea to render an individual medusa 'interstitial cell -free' (i-cell free) where the interstitial cell population is reduced to a very low level, and thus unable to form the normal progeny (Bode *et al.*, 1976). Fifteen jellyfish, with a diameter of less than 1 cm, were subjected to 3 to 5 cycles of a sequential treatment of a 24 hour exposure to 10^{-2}M solution of hydroxyurea in seawater, followed by a 12 hour cycle in normal sea water. In *Hydra*, this treatment has been found to eliminate the interstitial cells which have a cycle time of approximately 24 hours, without severely affecting the epithelial cells, which have a cycling time of approximately 72 hours (Bode *et al.*, 1976). After each of the third, fourth, and fifth cycles, the bell margins, including tentacles above the ocelli, of 5 animals were macerated in a solution containing glycerin: glacial acetic acid: water (1:1:26) at room temperature according to David (1973). When tissue pieces were completely dissociated, the resulting cell suspension was fixed by addition of formaldehyde to the cell suspension to a final concentration of 2% formaldehyde. Fixed cell suspensions were mixed with a drop of detergent (1% Triton X 100), spread on gelatin-coated microscope slides, and allowed to dry. The number of epithelial cells and interstitial cells was counted using phase contrast optics. Treatments of medusae for 3 cycles of hydroxyurea reduced the number of interstitial cells to a 50% state (Table 3.2). All experimental animals however, underwent 5 cycles of hydroxyurea treatment to ensure the most complete removal of interstitial cells.

Table 3.2 Interstitial Cell Population after Hydroxyurea treatment

Number of Hydroxyurea cycles	Interstitial Cell Density*
3	0.17
4	0.06
5	0.02
Control	0.32

* The animals were allowed to recover in normal seawater for three days after the hydroxyurea treatment before they were macerated. I-cell density was measured as the ratio of interstitial cells to epithelial cells in the first 1000 cells counted per sample.

In *Hydra*, 5 treatments have been found to be most successful in rendering the animals 'i-cell free' (Bode, pers. comm.).

Interstitial cells were removed from medusae (<24 tentacles) with tentacle bud stages 1-4, and from animals which had had stage 2 buds removed by extirpation. Only stage 2 buds were used to examine regeneration in the absence of interstitial cells because these buds are readily identifiable, and they regenerate well in culture. The hydroxyurea experiments for both ontogeny and regeneration were replicated three times over a period of 3 months. Five animals per stage were used to observe ontogenetic changes in the tentacle bud stages 1-4 (n=20), per replicate of the experiment (n=60). To examine regeneration over a 30 day period, an additional 10 individuals with stage 2 buds removed were used per replicate of the experiment (n=30). Prior to the hydroxyurea treatment, each animal was examined and drawn, and in the case of the regenerates, their buds were extirpated and marked as described above. During the treatment, the animals were kept in standing water cultures in incubators, at 12°C, with a 12 hr day/12 hr night light cycle. After the treatment, the animals were put through several changes of normal sea water, and then transferred to the standing sea water cultures described previously. The animals were observed daily, and any morphological changes were noted. When any change in developmental stage (from stage 1-4) was noted, the animals were fixed and stained for RFamide as described previously. Once a week during the course of all the experiments, 2 additional individuals were fixed in absolute ethanol, and stained with toluidine blue to ensure that the animals had not regained their interstitial cell population (a total of 24 animals for three replicates of the experiments). Pieces of the bell margin were fixed 1hr in 100% ethanol, and then placed in PBS (phosphate buffered saline) (Diehl and Burnett, 1964). The pieces were stained in 0.05% toluidine blue in PBS (pH 7.8) for 1-2 minutes. The bell margin pieces were then rinsed in PBS and destained in 70% ethanol. The bell margin pieces were mounted in Mowiol (Calbiochem 4-88, see Chapter 2), and examined

for the presence of interstitial cells. This staining was done as a precaution to ensure that the i-cell population remained reduced throughout the experiment. Bode and David (1978) found that it took approximately 40 days for the i-cell population to recover in *Hydra* .

Results

Timing of tentacle development during ontogeny

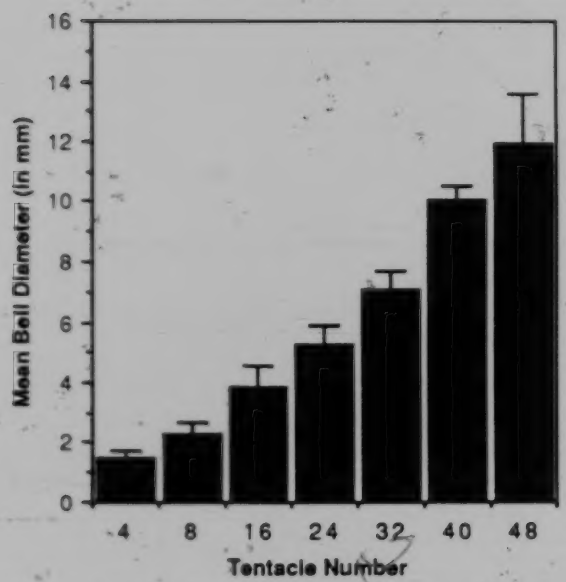
The timing of tentacle formation is somewhat variable in culture. A developmental timetable is shown in Table 3.3. The growth of tentacles varies with the size of the medusa, such that a four tentacle medusa changes to an eight tentacle medusa more rapidly than a twenty four tentacle medusa changes to a thirty two tentacle medusae. Once an ectodermal bud has formed, the endoderm will evaginate within 3-6 days, and an ocellus will form in 2-8 days. The elongation of the bud usually begins during, or immediately after, ocellus formation, and the tentacle can attain full length in 2-10 days (Table 3.3). Full tentacle length was defined as the point when the developing tentacle was the same length as the adjacent, older tentacles (in animals < 16 tentacles) or was contracting synchronously with the mature tentacles. In culture, the sixteen tentacle stage seems to be quiescent for some time with respect to tentacle growth, remaining in this stage for more than a month (n=12). Initially it was thought that there was a large increase in size between the 16 and 24 tentacle stages, but measurements of bell diameters versus tentacle number showed continuous increase in size throughout tentacle development (n=190) (Figure 3.1). A number of unique morphological changes occurred between the sixteen and twenty four tentacle medusa stages which defined the medusa as an adult *Polyorchis penicillatus*. These changes were: the development of diverticulae on the radial canals; the appearance of gonads; the loss of the cnidothylacie from the aboral surface of the bell; the development of fringed lips on the manubrium; and the development of a peduncle. These developmental timetables were constructed to provide a baseline upon which the regeneration experiments involving tentacle development could be compared.

Table 3.3 Rate of Development in Culture

Tentacle no.	Length of time in each stage at 12 °C			
	Stage 1	Stage 2	Stage 3	Stage 4
4 tentacle	1 day	2-5 days	1-5 days	2-4 days
n=16		avg 3 days	avg 2 days	
8 tentacle	1 day	3-10 days	1-9 days	2 days
n=21		avg 5 days	avg 4 days	
16 tentacle*	1-4 days	4-10 days	2 -17 days	5-7 days
n=20	avg 3 days	avg 6 days	avg 8 days	avg 6 days
24 tentacle	2-5 days	2-9 days	4-6 days	5-11 days
n=14	avg 4 days	avg 5 days	avg 5 days	avg 7 days
32 tentacle	2-5 days	3-9 days	4-6 days	2-10 days
n=8	avg 4 days	avg 6 days	avg 4 days	avg 4 days

*The timing given under 16 tentacle stage is for medusae in this stage which actually form buds in culture, and once the buds have begun to form. 60% (12/20) of medusae kept in culture at this stage never formed buds for the next whorl, even after 2 months in culture. Other morphological changes did occur however (see text).

Figure 3.1 Bell Diameter as a Function of Tentacle Number



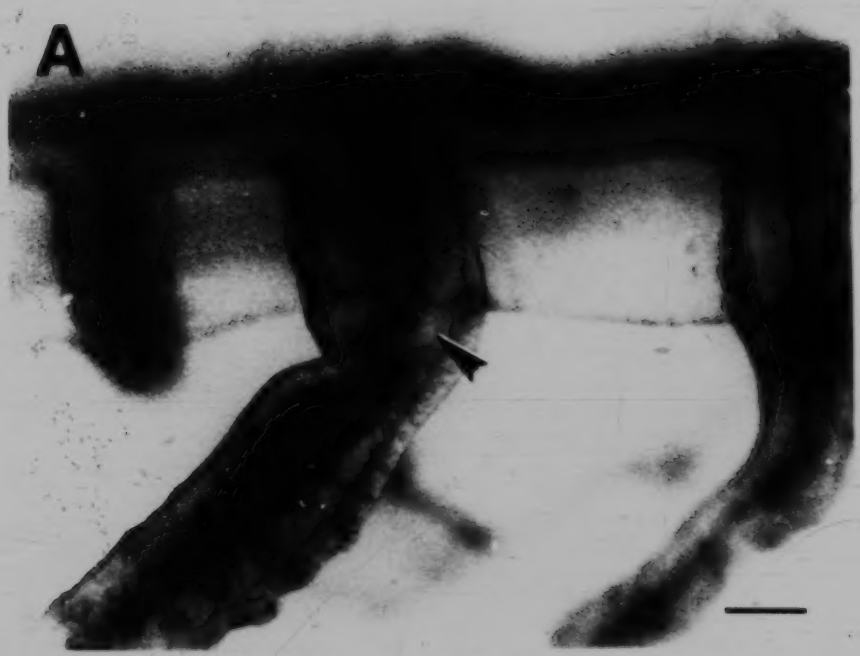
Rate and extent of regeneration of bud stages

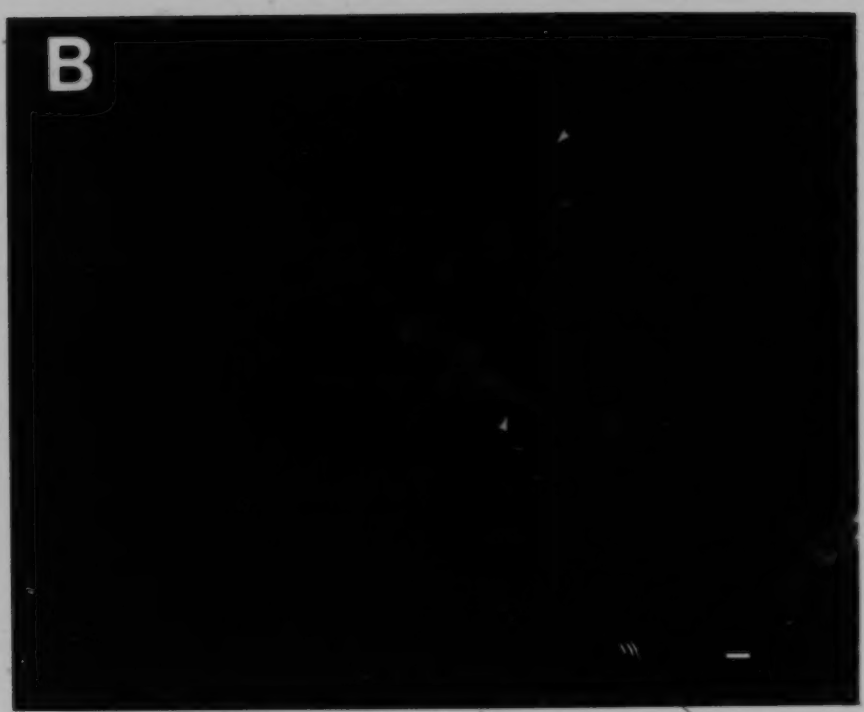
Wounds produced by extirpation of tentacles heal within an hour of the operation. In 'young' medusae (< 24 tentacles) Stage 1-4 buds, as well as mature tentacles, which have been removed by extirpation will regenerate as a Stage 1 bud and complete tentacle formation in the same way as a bud formed during ontogeny. The time between stages during regeneration is increased as compared to ontogeny (Appendix II). Tentacle buds removed from older medusae regenerate in the same way, but require more time to regenerate than do buds extirpated from younger medusae. In larger medusae (> 24 tentacles), tentacle regeneration does not generally occur at any stage, although there is sometimes initial development of a stage 1 bud which later disappears (Appendix II). The other buds of the same whorl as the extirpated bud do not stop developing when the experimental bud is extirpated. Often the next whorl of buds has already begun forming before the extirpated bud has reached stage 3. The regenerating bud is developmentally retarded when compared with the other buds of its whorl until the regenerated bud has formed a tentacle rudiment.

Formation of ocellar pigment cells after extirpation is often severely delayed, and when the ocellus itself is regenerated, the pigment cells may only be partially replaced. Indeed, the regenerated bud, unlike a stage 3 bud developing during ontogeny, may elongate into a tentacle rudiment without pigment cells having formed. When only the ocellus is removed from a mature tentacle or a tentacle rudiment, pigment cells of the ocellus are not replaced. Older medusae, found in the water column, are often found to have pigment cells missing from one or two tentacles (Plate 3.1a). When these tentacles are stained for RFamide, the ocellar nerves, and the large ocellar cells are still both present, and immunoreactive, despite the absence of the pigment cells (Plate 3.1b).

Immunofluorescence of bud regenerates stained with RFamide shows virtually no difference in staining pattern as compared to bud stages which develop during ontogeny

Plate 3.1 A. A mature tentacle lacking ocellar pigment found on a medusa collected by plankton tow. Scale bar=0.5mm. B. Despite the lack of the ocellar pigment cells, the RFamide immunoreactive ocellar nerves, and cell bodies remain intact in a mature tentacle. The ocellar nerve is labelled with an arrowhead. The tentacles in A and B are not the same tentacles, although tentacles lacked ocellar pigment cells. Scale bar=0.1mm.


A


B

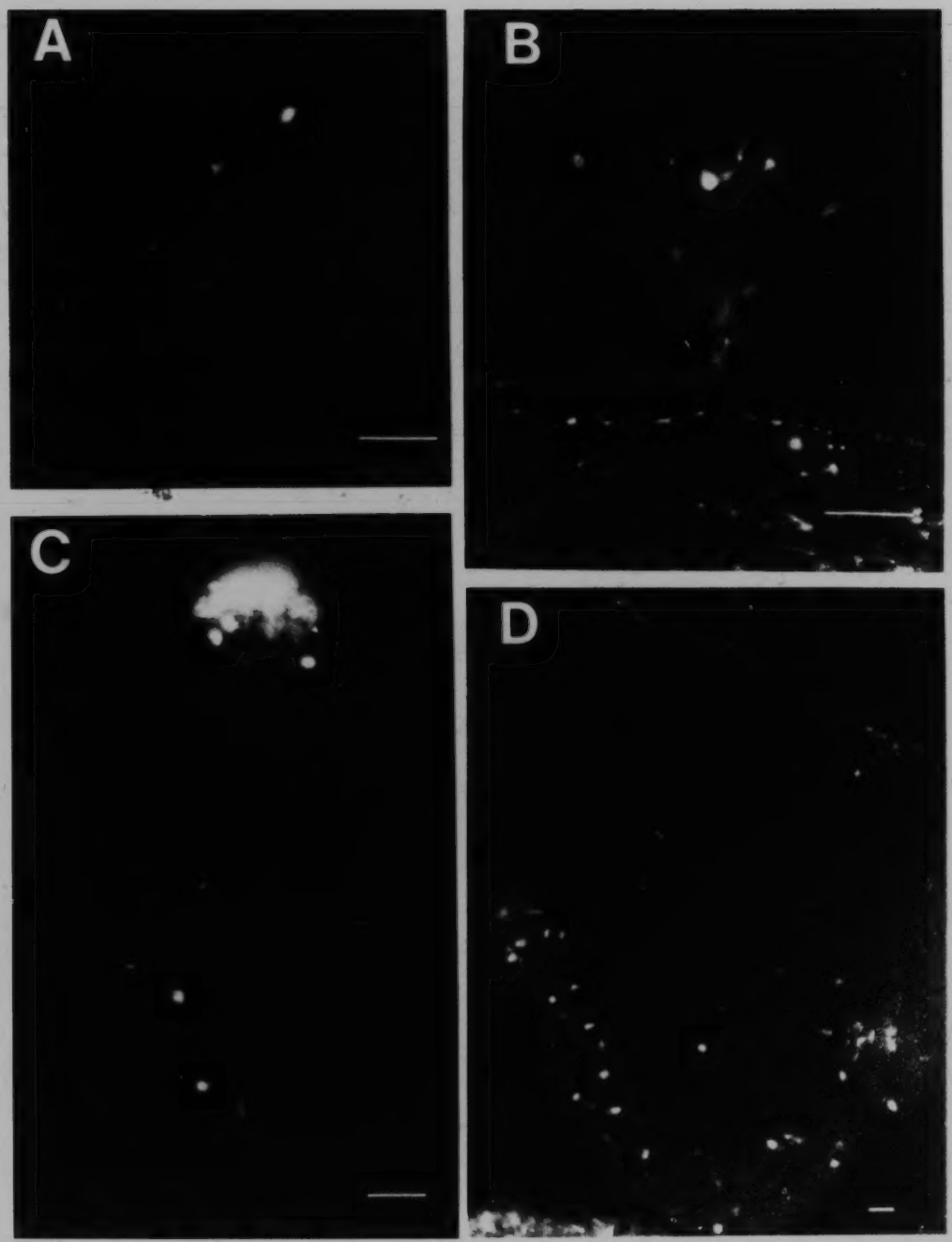
(Plate 3.2). In Stage 1, one or two immunoreactive cells are seen in the intertentacular region (n=10). In Stage 2, a cup like arrangement of 12-16 neurons is seen (n=10). A few large immunoreactive cells are seen in the bud apex (n=10) in Stage 3, even if no pigment cells are regenerated. Lastly, with the regeneration of the tentacle rudiment, an immunoreactive nerve net was seen (n=8). No increase in the number of immunoreactive neurons seen in the regenerative situation was seen over the the number of such neurons seen during ontogeny.

Effects of interstitial cell removal during tentacle ontogeny and regeneration

After undergoing hydroxyurea treatment the medusae appear normal although their tentacles have very few nematocysts. Stage1 and Stage 2 buds which were subjected to the hydroxyurea treatment did not show any further development during the month long observation period after the hydroxyurea treatment (100% of the buds examined; n=15/stage). Some stage 3 (ocellus; 33% or 5 animals) and Stage 4 (tentacle rudiment; 53% or 8 animals) buds however, did show elongation into tentacle rudiments in the observation period subsequent to the hydroxyurea treatment. When stained with anti-RFamide, these stage 3 and stage 4 buds which had elongated during the experiment showed a normal distribution of cells stained with anti-RFamide.

In all three replicates of the experiment, the regenerates which had had a stage 2 bud removed by extirpation, did not show any signs of regeneration of the bud (n=30, 100%). The wound was allowed to heal one hour before the animal underwent the hydroxyurea treatment, but no development of even an initial bud was observed. The tentacles on either side of the extirpated bud, which had been trimmed to mark the site of extirpation, did, however, show elongation in most circumstances (60%, or 18 animals showed this elongation). Staining of the extirpated Stage 2 buds with anti-RFamide showed no development of the RFamide positive cells characteristic of Stage 1 or Stage 2 buds (See

Plate 3.2 RFamide-like immunoreactivity of regenerated tentacles, in the different bud stages. The stages which the regenerated tentacle passes through are the same as those seen during tentacle ontogeny, and regeneration occurs with very high fidelity. A. Regenerated bud in stage 1, viewed from the ONR side. Scale bar=40μm. B. Regenerated bud in Stage 2, viewed from the ONR side. Scale bar=50μm. C. Regenerated bud in stage 3, viewed from the ONR side. Scale bar=40μm. D. Regenerated tentacle rudiment, viewed from the INR side. Pigment cells at the ocellus are largely absent, but the immunoreactive ocellar nerves and cell bodies are present. Scale bar=30μm.


A
B
C
D

Chapter 2). The intertentacular plexus of immunoreactive profiles was intact however, and the long tentacles which were trimmed and regrew, showed a normal staining pattern with anti-RFamide. The weekly staining of two animals with toluidine blue throughout the course of all the interstitial cell removal experiments did not reveal the presence of any interstitial cells in the first few weeks of the experiments, and in the later weeks of the experiments, only a few interstitial cells were visible (15% of the intial number of interstitial cells seen by toluidine blue staining).

Discussion

The timetable of tentacle ontogeny was constructed as a baseline upon which tentacle development in medusae with regenerating tentacles and medusae treated with hydroxyurea, cultured under the same conditions could be compared. The duration of each developmental stage (Stage 1-4) was variable with the age of the medusae, both between individual medusae of the same age, and even between quadrants of a single individual. Due to these limitations, the timetable of tentacle development in culture is at best approximate.

Regeneration of tentacle buds and tentacles did occur to some extent in *Polyorchis*. In younger animals (<24 tentacles), tentacles, tentacle buds, and ocelli were replaced. Older animals, however, did not tend to replace lost parts. This full regeneration of tentacles in mainly younger animals suggests an age-specific capacity for regeneration. All organisms must presumably allocate a limited amount of energy to various biological functions such as growth, reproduction, regeneration and maintenance. Perhaps in younger animals the need to replace lost parts is more important for survival, so more energy is allocated to regeneration. This phenonemon of age-specific capacity for regeneration is seen in other phyla. For instance, frog larvae have the capacity to regenerate the hind limbs but lose this capability at metamorphosis (Schotte and Harland, 1943; Van Stone, 1955). Also, the ability to regenerate appendages in adult insects is lacking or incomplete, however during larval and pupal stages the ability to regenerate is considerable (Needham, 1965). There are so many exceptions to this age-specific capacity for regeneration that it cannot be regarded as a general rule, although it is thought that many

adult tissues contain negative feedback inhibitors of mitosis called 'chalones' which may result in a decreased ability to regenerate (Thornley and Laurence, 1975).

In comparison to other medusae and to polyps, the regenerative capacity of *Polyorchis* was limited. Another anthomedusa, *Cladonema radiatum*, is able to completely regenerate an extirpated ocellus, although there are some differences between ontogenetic and regenerative differentiation of the pigment cells (Weber, 1981). In *Polyorchis* however, regeneration of the pigment cells of the ocellus was at best difficult, usually incomplete, and often did not occur, although the RFamide-positive neurons of the ocellus did regenerate. Schmid and Tardent (1971) found that the leptomedusa *Phialidium hemisphaericum* could essentially reconstitute a whole medusa from a fragment of the umbrella which lacked any components of the gastrovascular cavity or bell margin, and was therefore free of interstitial cells. A similar situation exists in the anthomedusa *Elutheria dichotoma* which can replace practically all amputated organs as long as part of the original structure remains (Weiler-Stolt, 1960). The regenerative potential of the anthomedusa *Podocoryne carnea* is also extensive, although a gradient of regenerative capability was found to exist in the medusa (Schmid, 1974). This gradient decreased from the centre towards the periphery of the animal (Schmid, 1974). Unfortunately Schmid (1974) did not examine the regenerative potential of the bell margin itself and thus the regenerative capacity of its tentacles or neurons in the tentacles is not known. It may be that the cells of the bell itself are in fact more simple and less specialized than the cells of the bell margin and the tentacle proper. In this situation, the cells of the bell might exhibit only a limited degree of functional differentiation, whereas with the formation of more specialized organs such as tentacles, the cells have greater functional differentiation, and the organism locally sacrifices some of its regenerative abilities in order to have this greater specialization. In this situation, which would concur with Schmid's (1974) gradient, the

power of regeneration would be inversely proportional to the degree of tissue differentiation.

Medusae on the whole, even those with greater regenerative capabilities than *Polyorchis*, do not show the regenerative capacity of *Hydra* and other hydroid polyps. Medusae demonstrate a higher level of organization and cellular differentiation than polyps and it has generally been assumed that the less differentiated polyp has a greater regenerative potential than the more highly differentiated medusa. *Hydra* is capable of completely regenerating both head and foot structures, and of reaggregation after dissociation (Gierer *et al.*, 1972; Bursztajn and Davis, 1974; Bode *et al* ., 1986; Müller *et al.*, 1986). Other hydroids show regenerative capabilities similar to *Hydra*. *Tubularia* and *Corymorpha* regenerate head structures as do the polyps of *Hydractinia echinata* (Berrill, 1961; Tardent, 1963; Rose, 1970; Müller, 1969; 1982; 1985). Another hydractiniid hydroid *Podocoryne carnea* regenerates both head and foot structures (Müller *et al.*, 1986).

Regeneration results in the reestablishment of both normal gross morphology and normal asymmetrical cell distributions. Tentacle regeneration in *Polyorchis* occurs with very high fidelity. The newly formed structure resembles the original tentacle with regard to the position of the RFamide-positive neurons, and in the overall tentacle morphology, except for the pigment cells of the ocellus. The regeneration of a tentacle bud at any stage or of a mature tentacle proceeded through the same stages as seen in the ontogenetic development of the tentacle, no matter what stage the tentacle bud had been prior to extirpation. Initially, there is an outpocketing of ectoderm in the intertentacular region where the bud was removed. Subsequently, there is an outpocketing of the endoderm. Ocellar pigment cell formation sometimes occurs, but the regenerating tentacle, in contrast to ontogenetic development, often elongates into a tentacle rudiment without forming any pigment cells. This pattern of regeneration is typical of the Hydrozoa where regeneration

often parallels the process of budding (Hay, 1966). Regeneration of the RFamide system, where regeneration of the tentacle did occur, was complete, and indistinguishable histologically from the RFamide system of neurons which developed during tentacle ontogeny (Chapter 2) even when regeneration of the ocellus was incomplete. Similarly in *Hydra* it has been found that exact and allometric proportioning of the cells and gross morphology of the regenerating tissue is maintained (Bode and Bode, 1984).

It has been suggested that control of regeneration may be mediated by the neurosecretory substances released by neurons (Lentz and Barnett, 1963; Burnett *et al.*, 1964; Lentz, 1965a,b) or by a threshold number of neurons being present in the regenerating tissue (Singer, 1952), and producing some as of yet unknown trophic factor, irrespective of the type of neurons present. In either case, one might predict an increase in RFamide-positive neurons in the regenerating tentacle buds of *Polyorchis* if these neurons are responsible for inducing regeneration. Bode *et al* (1973) did find that *Hydra* produced more neurons at a regenerating tip than this same region would produce in a normal animal. However in *Polyorchis*, no increase in the number of RFamide neurons innervating the regenerating bud was seen over the number of RFamide neurons innervating the bud in the ontogenic situation. This lack of increase in the number of neurons does not preclude the possibility that neurons in general, or RFamide-positive neurons specifically, induce regeneration as it is not known whether the number of RFamide-positive neurons seen in both developmental and regeneration situations reach a 'threshold' of neurons required for tentacle bud formation. Also there are some limitations on immunofluorescence in light microscopy, since neurons which are not yet synthesising neuropeptides might not be labelled, but are already committed to being RFamide-positive neurons. As well, if RFamide peptides are neurosecretory substances which induce regeneration, the same number of cells may synthesize and release more RFamide in the regenerating situation, without one seeing an increase in immunofluorescent signal. Lastly, other RFamide-

negative neurons innervating the developing or regenerating bud, but unfortunately not visualized, may be responsible for attaining a threshold number of neurons in either situation, or indeed these neurons might be increased during regeneration, but remain undetected.

Hydroxyurea effects during ontogenetic and regenerative tentacle development

During the ontogeny of tentacles in *Polyorchis*, RFamide-positive neurons are present in the tentacle buds at a very early stage in development (Stage 1, see Chapter 2). Hydroxyurea treatments of the early developmental stages (Stages 0-2) to remove the interstitial cells which would form neurons at these stages, resulted in cessation of further neural development, and also of the development of the tentacle at that stage. Later stages of tentacular development (stage 3 and stage 4) seem to be less affected by hydroxyurea treatment. In these later stages the ocellus has already formed and the tentacle needs only to elongate to form a mature tentacle. The only neurons which remain to be formed are those neurons in the ectodermal nerve-net of the tentacle. Elongation of the tentacle during ontogeny occurred normally after hydroxyurea treatment, and the RFamide-positive neurons of the ectodermal nerve-net in the tentacle also appeared normal. Also, although regeneration of stage 2 buds did not occur at all after hydroxyurea treatment, those tentacles which had been trimmed to mark the extirpation site did regrow after hydroxyurea treatment. These regenerated tentacle tips also appeared normal with respect to the tentacle morphology and the RFamide-positive nerve-net in the tentacle. In both of these situations the only tissue to regenerate or develop subsequent to hydroxyurea treatment were tentacles below the ocellus, and their RFamide-positive ectodermal nerve-net. Both the manubrium and tentacles of *Polyorchis* and other medusae have an ectodermal nerve-net similar to *Hydra* . In regeneration of *Hydra* , where the neurons form an ectodermal nerve-net, regeneration of the animal and neurons occurs independently of the presence of interstitial

cells (Diehl and Burnett, 1964; Burnett *et al.*, 1964). Webster and Hamilton (1972) found in *Hydra* that elongation of extant buds was possible after the removal of neuronal precursors, but that initiation of new buds was not possible. The absence of interstitial cells is also of minor importance in the regeneration of the manubrium in *Podocoryne* (Schmid, 1974).

There appears to be some difference in the type of neurons which are able to grow or regenerate after hydroxyurea treatment. A differential effect of hydroxyurea treatment on neurons has also been seen in *Hydra* and in larvae of *Phialidium gregarium* and *Halocordyle* (Yaross *et al.*, 1985; Bode *et al.*, 1986; Thomas *et al.*, 1987). In *Hydra*, no new neurosensory cells are formed, but the nerve-net is left unaffected (Yaross *et al.*, 1985) however, in *Phialidium* and *Halocordyle*, the neurosensory cells are retained and the 'ganglion'-type (multipolar) cells are lost (Martin and Thomas, 1981; Thomas *et al.*, 1987). One must consider where the RFamide-positive neurons which are formed subsequent to hydroxyurea treatment in the ectodermal nerve-net of *Polyorchis* came from. One possibility is that they arise from the very small number of interstitial cells which did not succumb to the hydroxyurea treatment. Although possible, this seems unlikely since tentacle development or regeneration subsequent to hydroxyurea treatment occurred in some stages only. The uniformity of regeneration and development would also tend to discount this possibility. Another possibility is that neurons from the ectodermal nerve-net of another region of the bell margin migrated into the newly forming tentacle. Although this seems unlikely, long distance migration of both interstitial cells and neurons has been documented in *Hydra* (Campbell, 1974; Herlands and Bode, 1974a; 1974b; Heimfeld and Bode, 1984a; 1984b). Long distance migration of interstitial cells in planula larvae of *Pennaria tiarella* has also been noted (Martin and Archer, 1986). An additional possibility is that cells of other types transdifferentiated into neurons of the nerve-net. Although unusual, transdifferentiation does occur, and has been documented in newt limb

regeneration, Wolffian lens regeneration, as well as in cnidarians (Reyer, 1954; Salpeter and Singer, 1960). In *Hydra*, RFamide-negative neurons of the nerve-net can transdifferentiate into RFamide-positive neurons in the absence of interstitial cells, however other subsets of neurons such as those neurons labelled by the monoclonal antibody JD1 can not be formed by transdifferentiation, and are lost when interstitial cells are removed (Yaross *et al.*, 1985; Bode *et al.*, 1986; Koizumi and Bode, 1986). In hydrozoan medusae, it has been found that transdifferentiation will occur *in vitro* . Smooth muscle cells will transdifferentiate into FMRFamide-positive cells, when the cell layers have been separated from the extracellular mesoglea by collagenase treatment (Alder and Schmid, 1987). In this culture system it has also been found that a regenerate will form from the smooth muscle and FMRFamide-positive neurons. As the regenerate forms the number of FMRFamide-positive neurons decreases, and it is therefore thought that the FMRFamide neurons in this instance may play a role in the organization of the regenerate (Alder and Schmid, 1987). One final possibility is that the the neurons of the ectodermal nerve-net arise from a different type of stem cell which is either not affected by the hydroxyurea treatment or is an interstitial cell which has a different cycling time. The possibility of another type of stem cell, however, does not fit with data found for interstitial cells in neuronal differentiation of *Hydra* and other hydrozoans.

The presence of neurons has been implicated as an essential component for development and regeneration in many phyla (Child, 1920; Needham, 1952; Singer, 1952; Zalewski, 1969; 1970). In the Hydrozoa, neurons and neurosecretion have been implicated in such processes as cell differentiation, budding, sexuality, and regeneration (Lentz and Barnett, 1963; Burnett and Diehl, 1964; Lentz, 1965a,b; 1966; Davis *et al*, 1968; Bursztajn and Davis, 1974). Ham and Eakin (1958) found an increase in the number of regenerating tentacles following treatment of regenerating *Hydra* with a variety of neuropharmacological agents. Similarly, Lentz (1965b) found that excised midsections of

Hydra exposed to isolated neurosecretory granules developed additional heads as they regenerated. The present study of the effect of hydroxyurea on interstitial cells at different stages of tentacle development, and in regenerating tentacles, assumes that the formation of the neurons is coincident with, and necessary for, the development and/or regeneration of the tentacle. In *Polyorchis*, the presence of neurons early in the development of the tentacle has been seen (Chapter 2), but the necessity of their presence in terms of bud development and regeneration has not been demonstrated. There are several problems with the hydroxyurea experiments. It is possible that the treatment has some other effect which is not observed, but which causes inhibition of development or regeneration at certain stages, with no relation to the removal of the neuronal precursors. An additional difficulty is that only RFamide-positive neurons are seen histologically, and no satisfactory method has been found to label other neuronal populations of the tentacle (See Appendix I). One further thought is that it does not seem that the hydroxyurea experiments satisfactorally differentiate between the importance of the presence of interstitial cells and the importance of neurons since both are eliminated. Earlier work had assumed that neurons were obligatory, but it has been found that epithelial *Hydra* , devoid of both interstitial cells and neurons are capable of budding, regeneration and polarity reversal without the presence of either interstitial cells or neurons (Marcum *et al.*, 1977; Marcum and Campbell, 1978). It seems likely that cellular interactions during growth and regeneration are complex. In *Hydra oligactis* inhibition of collagen secretion with the proline analog L-azetidine-2-carboxylic acid inhibited tentacle regeneration without inhibiting the nerve cell differentiation that normally precedes tentacle growth (Lesh-Laurie *et al.*, 1986). Shostak (1982) found that there was a correlation between structural cell number and tentacle regeneration and development. Therefore for regeneration of a tentacle to occur a certain mass of tissue was required, and during development a certain number of cells had to be present before tentacle buds could form (Shostak, 1982). Tardent (1963) found that the

endoderm has the predominant role in establishing organogenetic patterns, and that tentacle differentiation is induced by the underlying endoderm. Further, he found that interstitial cells played no organizing role in development or regeneration (Tardent, 1963).

If neurons do play a role in development and regeneration, there are several possibilities as to the extent of their involvement in these processes: (i) neurons may not be involved in tentacle development at all, and some other process may be occurring to inhibit growth and regeneration during the hydroxyurea treatments; (ii) neurons may play a role in fine tuning developmental patterns which are basically established by other cells or processes; or lastly, (iii) neurons may play an essential role in establishing tentacle growth and regeneration in *Polyorchis* , but not be as important in the later stages of tentacle formation.

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IV. Conclusion

The purpose of this thesis was to examine the RFamide-immunoreactive neurons in the hydrozoan medusa *Polyorchis penicillatus* during growth and regeneration in an attempt to ascertain possible functions of these neurons. I have done this using a variety of techniques and approaches. In this final chapter I will briefly review some of the major findings of each chapter, and relate these findings to each other. Further, I will identify areas of further research, and the findings and problems this research might reveal.

In Chapter 2, I described the stages of tentacle development in *Polyorchis*, in terms of the gross morphology of the tentacle and the morphology of the RFamide-immunoreactive neurons. The development of the 'B' system of neurons in the tentacle was indirectly compared to the RFamide-immunoreactive neurons at the different tentacle stages. One major problem of examining the neurons of the different tentacle stages was that no satisfactory method was found for labelling the other systems of neurons (the 'B' and 'O' systems) which are known to enter the mature tentacle. As well, there may be other unknown neuronal subpopulations which have been ignored in the present study simply because of an inability to visualize the neurons. Classical silver staining techniques have been used with some success on cnidarian tissue despite the fact that cnidarian neurons are unmyelinated. Such silver techniques however, are notoriously 'fickle' and it is known that only a subset of the total number of neurons will be stained during a particular staining procedure. Silver staining techniques are therefore not very useful for developmental studies since one important factor in such studies is the constancy of staining at each stage. Another technique which was attempted in preliminary studies is to iontophoretically fill neurons in the tentacle with the fluorescent dye Lucifer Yellow. There

are two problems with employing this technique in *Polyorchis*, the first is that the neurons in *Polyorchis* , are small and technically very difficult to fill; the second problem is that only a very small percentage of neurons in a tentacle can be labelled. The optimal method of labelling neurons in the tentacle buds would be to develop monoclonal antibodies to the various subpopulations of neurons in the tentacle bud. I have made numerous attempts over the past two and a half years to develop monoclonals to the different neuronal subpopulations in the tentacles of *Polyorchis* without success (See Appendix I). Other researcher's attempts to develop such monoclonals against *Polyorchis* neurons have been similarly thwarted. Given the apparent difficulty of developing such antibodies, this method of labelling may be ideal, but in truth not very practical at this time.

Another method of examining the developmental role of the RFamide peptides in *Polyorchis* is to label the peptides with immunogold at the electron microscope level. I was unsuccessful at obtaining specific labelling (See Appendix III), however recently Singla (unpublished data) has successfully labelled dense-cored vesicles in *Polyorchis* with RFamide. By employing this powerful technique much information can be gained about the possible roles of RFamide. One of the first things to be done is to determine the location of the RFamides in the various subpopulations of neurons which were outlined in Chapter 2. The ONR, the INR, the immunoreactive cells of the nerve-net and of the ocellus, and those immunoreactive cells present in Stage 1 and Stage 2 buds, should all be examined with this label to determine where the vesicles are located, whether the vesicles are at synapses or not. If the labelled vesicles are located at synapses the type of synapse should also be identified (ie. neuro-neuronal, neuro-muscular). Observations from light microscopic immunofluorescence of beading along fine processes that parallel smooth muscle fibres is suggestive of synapses, and would fit in well with the conceptual model for the innervation of the nerve-net of the tentacle described in Chapter 2 (Figure 2.5). If the vesicles are not located at synapses, as may be the case in regions where the peptide has

a neuromodulatory or neurohormonal role, the identity of potential target cells should be determined, this identification is difficult without the presence of synapses, but adjacent cells could be identified on the assumption that the peptides act over a fairly short distance. The type of target cell, and its RFamide receptor, could be examined by culturing the different cell types, and then characterizing the receptor molecules by radioiodination of Pol-RFamide with ^{125}I on a free amino group. It should also be confirmed that the RFamide-positive neurons are distinct from the 'O' and 'B' systems. One technically difficult way of doing this would be to fill cells in culture with Lucifer which have been electrophysiologically characterized as 'O' or 'B' neurons. The identified neuron would then be embedded and examined in the electron microscope. A double immuno-staining technique against Lucifer and RFamide, employing two different sizes of gold particles, would be used to determine if the identifed neuron was also RFamide-positive. The physiological functions of the RFamide peptide could be addressed by treating the different identifed subpopulations of neurons (ONR, INR, ocellar cells, nerve-net cells, and the cells of Stage 1 and 2 buds) with high external K^+ solutions prior to fixation to depolarize the neurons and initiate vesicle release. The amount of label in the different neuronal subpopulations could be compared to a control in which the K^+ concentration was the same as in the fixative solution. Such an experiment would determine whether release of RFamide is depolarization-dependent as might be the case for a neuromodulator or neurotransmitter.

The possibility that the RFamide peptides are neurohormones or morphogens was suggested in Chapter 2. No one has yet addressed this possibility experimentally. One possible technique would be to incubate *Polyorchis* in sea water containing RFamide, and alternately anti-RFamide, to determine the effects, if any, on tentacle development and regeneration. There are several problems with this experimental design. Penetration of the peptides or the antibodies into the extracellular space might be difficult, and clearance or

degradation of the peptides or antibodies might be rapid. Also, tentacle development is not an ideal system for examining this problem, because tentacle development occurs fairly slowly in culture, and it may be difficult to determine any effects that the peptides or antibodies had on development. A system which might yield better results would be to culture the different types of cells, incubate these cultures in either the peptide or antibody solution, and examine the cells for mitogenic or changes in rates of synthesis. Depending on the stability of the solution, useful information might be obtained. Lastly, the possibility that RFamide peptides are acting as neurohormones in *Polyorchis* , might be examined by raising planula larvae, and exposing the planulae to exogenous RFamide peptides to see if metamorphosis is induced. However the same problems of exogenously applied RFamide also pertain to this experiment. If RFamide did cause metamorphosis of the planulae, the test would be rapid and definitive, although RFamide might act indirectly as a chemical stimulus causing release of the true morphogen.

Chapter 2 outlined the developmental process of tentacle growth, and the distribution of RFamide neurons during this process. Possible functions of the RFamide peptides, acting as neurotransmitter, neuromodulator, neurohormone, or morphogen were suggested. Chapter 3 attempted to identify a potential role of neurons in growth and regeneration of the tentacles. In Chapter 3, I determined the overall potential for tentacle regeneration, and compared the regeneration process of the tentacles in terms of gross morphology and the RFamide neurons to the development of tentacles from ontogeny. Further, I attempted to assess the importance of neurons for growth and regeneration, by chemically removing neuronal precursors from the tentacles, and then watching for further development. The regenerative potential of the tentacle in *Polyorchis* can be described as limited at best in comparison to other hydroids, and even other hydrozoan medusae. Thus, it might be more worthwhile employing these experimental techniques on a different species. Also, despite the vast amount of work using these techniques of interstitial cell

removal in *Hydra* and other hydroids, it is difficult to believe that other cell types are not affected by hydroxyurea or the other treatments which kill mitotically active cells. The effect of these treatments on the other cell types might somehow affect the growth or regeneration of the animal or the body part in a way which is not addressed in the present body of literature. Also, even if other cell types are not affected by the treatment, it is difficult to determine whether it is the neurons or the interstitial cells which are important for tentacle outgrowth, or growth and regeneration in the other systems which have employed this technique. Lastly, the researchers using the *Hydra* system as a model have always assumed that reducing the interstitial cells to a very low level renders the interstitial cells incapable of either differentiating into neurons and inducing growth or regeneration , or from entering the budding region or the regenerating area and inducing growth or regeneration themselves.

The specific functions of the RFamide-positive neurons remains elusive. Numerous studies have pointed toward several different functions, some of them contradictory. I have described the structural changes of the RFamide-positive neurons in the tentacles of *Polyorchis* during growth and regeneration. The other questions regarding the functions of *Polyorchis* RFamide-positve neurons can probably best be addressed through specific cytochemical localization of the RFamide peptides in the different neuronal subpopulations; the evidence of release of the peptide upon appropriate stimulus as determined by cytochemical localization; the physiological response of either the whole animal or cultured cells to exogenous application of RFamide peptides.

Appendix I- Production of Monoclonal antibodies against *Polyorchis* tissue

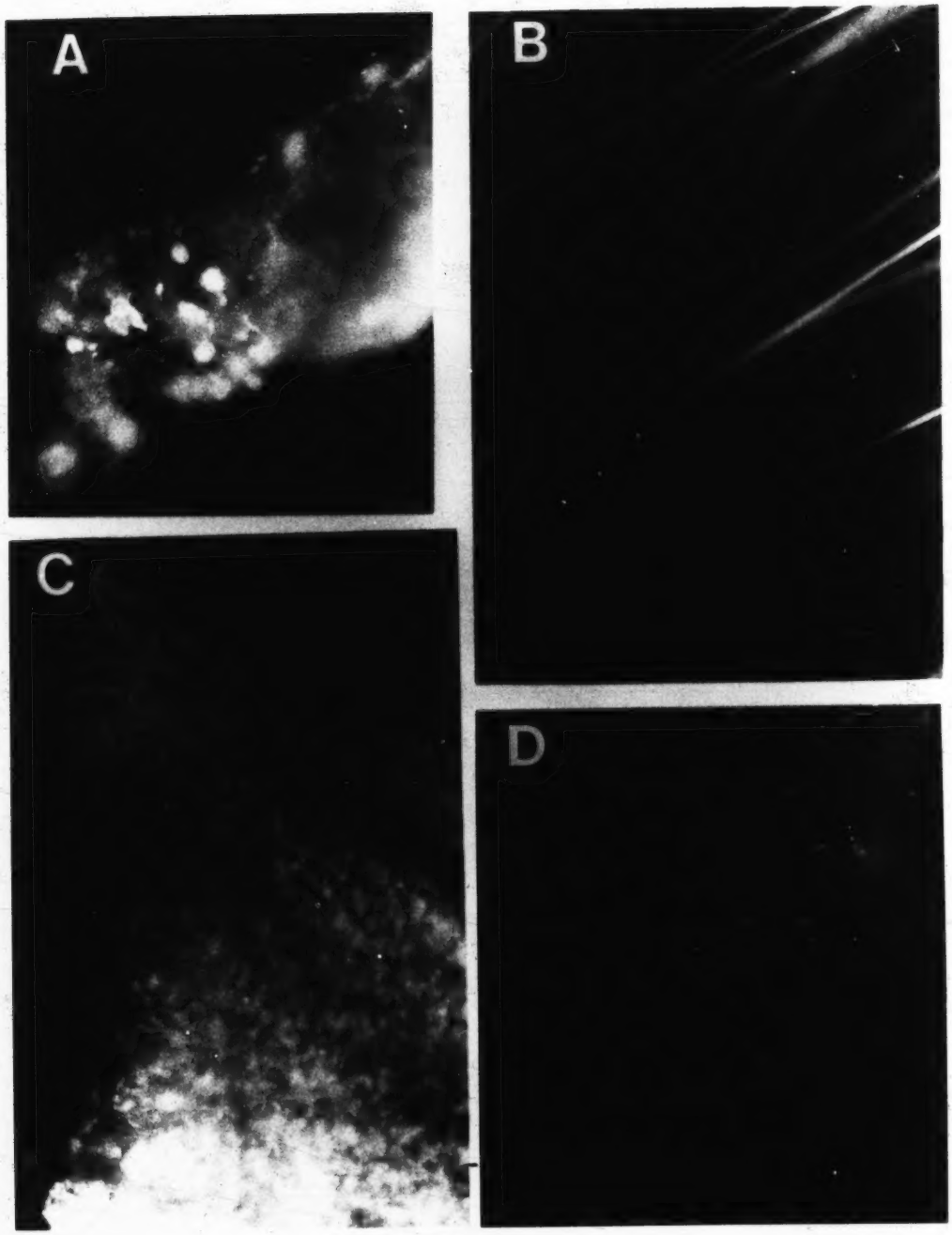
Numerous attempts, using several different methods, were made to develop monoclonal antibodies against neuronal subpopulations besides RFamide-containing neurons, in *Polyorchis*. Initially, a standard monoclonal antibody production technique was used. Female BALB/c mice were immunized with different innoculants (described later) at least three times at three week intervals. The first innoculant was made up with equal volumes of complete Freund's adjuvant. Subsequent injections were made up in incomplete Freund's adjuvant. The mice were tested to see if they were immmune by assaying the serum of blood obtained via tail vein bleeds. The mice, if immune, were boosted with antigen only, three days before the fusion. The mice were sacrificed by cervical dislocation. The spleen was removed using sterile technique, and the spleen cells were collected in medium. The red blood cells were lysed with 0.75% NH_4Cl solution, and the remaining white blood cells were washed several times in DMEM (Dubelco's modified Eagle's medium), counted and mixed in a 5:1 ratio with SP2/0 myeloma cells. The cell mixture was spun into a very tight pellet and the cells were fused with PEG (polyethelene glycol) to form hybridoma cells. The cells were washed and incubated overnight in DMEM at 37°C, and plated in a 96 well plate the following day, in selective HAT (hypoxanthine/aminopterin/thymidine) medium in which only hybridoma cells will survive. The cells were fed 5 and 10 days after feeding. Culture supernatants from positive wells were assayed after 12 days, and continued until no new wells were showing positive. Some wells were tested more than once. The assay technique used was the same as the staining technique used for staining for RFamide except that the primary antibody was the culture supernatant instead of antiserum 146II (See Chapter 2, methods). Both

positive (anti-RFamide) and negative (non-relevant supernatant, anti-chicken L-CAM (6H1) from Dr. Gallin) controls were included with the assays. The same assay system was used as it was important that any monoclonal antibody found could be used in the same system as 146II, so that double immunostaining would be possible. In some assays, a peroxidase-linked second antibody was used to eliminate autofluorescence, which tended to mask specific fluorescence in the assays.

Different types of antigenic material (innoculants) were used in different mice in an attempt to get an antibody response to neurons. Initially, bell margins including the velum and the tentacle above the ocelli, but lacking mesoglea was fixed in paraformaldehyde (4% solution in PBS) or left unfixed. The material was homogenized in a Dounce homogenizer before injection. Four fusions were performed using this innoculant. Another four fusions were performed using the velum only, which had been dissociated using Przysiezniak and Spencer's (1989) technique, as the antigenic material. The dissociated material was fixed in 4% paraformaldehyde, washed in PBS, spun down, and homogenized in a Dounce homogenizer prior to injection into the mice. In a third set of fusions, using another 4 mice, an attempt was made to tolerize the animals to non-neuronal tissue prior to their innoculation with the nerve-rich tissue. The mice were injected intaperitoneally (i.p.) with homogenized nerve-free tissue. Ten minutes later cyclophosphamide (Sigma (100mg/kg)) was given i.p. in 0.9% saline (2mg/ml). The same dose of the drug was given at 24h and 48h. This tolerizing treatment was repeated every 2 weeks, three more times. Following a three week rest, the mice were innoculated with nerve-rich homogenate at least three times, at three week intervals. The fusion was performed and assayed as described above.

Despite these variations in fusion protocol, no monoclonal antibodies to neuronal subpopulations were ever detected. However, monoclonal antibodies were made to epithelial cells, components of the mesoglea, and to cnidocytes (Plate A.1).

Plate A.1 Immunoreactivity of various monoclonal antibodies which were developed against *Polyorchis* tissue. A. Immunoreactive cnidocytes. Scale bar=30μm. B. Immunoreactive fibres of the mesoglea. Scale bar=20μm. C. Immunoreactive epithelial cells in the tentacle. Scale bar=10μm. D. Unknown immunoreactive cells, possibly interstitial cells of the tentacle. Scale bar=15μm.


A
B
C
D

As well as trying to make antibodies against other neuronal subpopulations in *Polyorchis* , I tried several different monoclonal antibodies and antisera which I either obtained commercially or recieved as a gift from other laboratories. I hoped that either there might be some cross-reactivity between species, or in the case of neurotransmitters, that immunoreactivity would be found. Some of the antibodies that I tried were anti-tyrosine hydroxylase (from Dr. Acheson); anti-dopamine (Incstar); anti-neurofilament (from Dr. Malhotra); anti thy-1 (Sigma); anti-vimentin (Sigma); anti-horse radish peroxidase (Sigma); anti-neuropeptide Y; anti-neuron-specific enolase (Zymed); anti-somatostatin and anti-salmon GnRH (Dr. Peter); MPM-2 and MPM-3 antibodies to cytoskeletal elements (Dr. Rao); and several *Hydra* antibodies from Dr. Bode: JD1 (stains sensory and 'ganglion' type neurons); RC-14 which stains the ectodermal nerve net; TS-33, an antibody to epidermal sensory cells; TS-26, a monoclonal antibody that binds 'ganglion' type cells; and CP-8 and TS-19 which stain epithelial cells of the head and tentacle region. Appropriate positive and negative controls were used in all experiments. No specific staining was noted with any of the antibodies that were tried.

In addition to immunochemical techniques, I tried histological staining techniques to stain other populations of neurons. Reduced methylene blue (McConnell, 1931) stained neurons, but the staining technique was unreliable, and different neurons were stained every time it was tried. Bielschowsky's silver method for dendrites and unmyelinated axons was also tried with similar results. In addition, acridine orange, which has been found to stain neurons in some invertebrates (Spencer, pers. comm.), was tried without success. We also attempted filling 'B' system neurons with lucifer *in vivo* and *in vitro*, and double labelling subsequently with anti-RFamide with equivocal results.

Appendix II- Rate of Regeneration in Culture

Stage at Extirpation	Length of time in each stage (avg for each treatment)			
	Stage 1*	Stage 2	Stage 3	Stage 4**
4 tentacle, **stage 1** n=4***	1 day	13 days	14 days	formed
8 tentacle, stage 1 n=4	2 days	15 days	17 days	formed
16 tentacle, stage 1 n=4	2 days	15 days	17 days	formed
24 tentacle, stage 1 n=4	2 days	14 days	13 days	formed
32 tentacle, stage 1 n=4	did not regrow			
big (> 64 tentacle), stage 1 n=4	did not regrow			
4 tentacle, **stage 2** n=4	1 day	14 days	10 days	formed
8 tentacle, stage 2 n=4	1 day	14 days	17 days	formed
16 tentacle, stage 2 n=4	2 days	16 days	17 days	formed
24 tentacle, stage 2 n=4	did not regrow			
32 tentacle, stage 2 n=4	did not regrow			
big, stage 2 n=4	did not regrow			
4 tentacle, **stage 3** n=4	1 day	23 days	6 days	formed
8 tentacle, stage 3 n=4	2 days	16 days	12 days	formed

* Buds generally took 1 to 2 days to begin reforming after extirpation.
** Tentacle rudiments were not always allowed to grow to full length, thus length of time in Stage 4 was not calculated, formation of tentacle rudiment was simply noted.
***n=4, does not include the two controls for each treatment.

Stage at Extirpation	Length of time in each stage			
	Stage 1*	Stage 2	Stage 3	Stage 4
16 tentacle, stage 3 n=4	2 days	9 days	23 days	formed
24 tentacle, stage 3 n=4	2 days	bud resorbed		
32 tentacle, stage 3 n=4	2 days	bud resorbed		
big, stage 3 n=4	did not regrow			
4 tentacle, **stage 4** n=4	no bud formed, 3 of 4 animals died			
8 tentacle, stage 4 n=4	2 days	15 days	10 days	formed
16 tentacle, stage 4 n=4	2 days	14 days	13 days	formed
24 tentacle, stage 4 n=4	2 days	13 days	12 days	formed
32 tentacle, stage 4 n=4	36 days (n=1)	did not regrow		
big, stage 4 n=4	did not regrow			
8 tentacle, **mature tentacle** n=4	2 days	13 days	14 days	formed
16 tentacle, mature tentacle n=4	2 days	17 days	22 days	formed
24 tentacle, mature tentacle n=4	2 days (n=2)	bud resorbed		
32 tentacle, mature tentacle n=4	did not regrow			
big, mature tentacle n=4	did not regrow			

Appendix III- Immunocytochemistry against RFamide peptides in *Polyorchis* tissue

Numerous attempts were made to label *Polyorchis* tissue with anti-RFamide at the electron microscopic level. Both pre- and post- embedding techniques were tried. Some preliminary results were obtained, but the staining was not present at synapses, as well there was non-specific backgound staining. Dr. Singla has subsequently been able to obtain specific labelling with anti-RFamide in *Polyorchis*, and we are now collaborating with him, and using his technique to examine the various classes of immunoreactive neurons in *Polyorchis*. What follows is an outline of some of the techniques which were used in attempting to label RFamide-immunoreactive neurons in *Polyorchis* at the E.M. level.

The first techniques tried were post-embedding techniques. A standard protocol was used for immunogold labelling. The tissue was fixed in either 1% glutaraldehyde in phosphate buffer, or in 4% paraformaldehyde in phosphate buffer or in artificial sea water at 4°C for 2h, sometimes the tissue was post-fixed in 1% OsO_4 in PBS or artificial sea water, but generally this step was omitted. The tissue was then dehydrated through an alcohol series to propylene oxide, although sometimes the tissue was placed directly into several changes of propylene oxide. The tissue was embedded in epon:araldite. Thin sections were cut with a diamond knife, and the sections were mounted on a 300 mesh nickel grid. For immunostaining, the grids were floated on successive drops of reagents at room temperature as follows: (1) osmicated tissue only was treated with saturated aqueous sodium metaperiodate for 20 minutes, rinsed well in double distilled water, and dried on fibre-free paper (2) 10% aqueous H_2O_2 for 3 to 5 minutes (sometimes not done) (3) 5 x 1 minute rinse on double distilled water (4) 15 min on normal goat serum diluted 1:20 with modified phosphate buffer (0.1 M sodium phosphate buffer, ph7.45, 0.5M NaCl, 0.5%

BSA and 0.05% Tween 20 or saponin); (5) 1h on RFamide antiserum diluted 1:1000 with modified phosphate buffer; (6) 3 x 5 min on modified phosphate buffer; (7) 1h on 15nm gold-conjugated, goat anti-rabbit IgG (Janssen-Life Sciences Products) diluted 1:50 with modified phosphate buffer (8) 3 x 5 min on modified phosphate buffer; and, (9) 2 x 5 min on PBS (0.15M NaCl, 0.01M sodium phosphate buffer, pH 7.45) After the final rinse the grids were postfixed for 15 min on a drop of 2% glutaraldehyde in PBS, and viewed unstained in a Philips-201 transmission electron microscope.

Variations to this method included altering the dilutions of antibodies, the type of fixative, and also the type of buffer. In addition, neurons were dissociated from the velum by the method described by Przysiezniak and Spencer (1989), the cells were fixed in either 1% glutaraldehyde in modified phosphate buffer or in artificial sea water (ASW), or in 4% paraformaldehyde in either of the two buffers. The cells were spun down into a pellet, and post-fixed in 1% OsO_4, washed in PBS or ASW and embedded in 3% agar in ASW or PBS. The agar blocks were then treated as tissue and prepared as described above.

Several pre-embedding techniques were also tried, where the antibody is applied to the tissue prior to embedding. A standard protocol was also used for the preembedding techniques. The tissue was fixed for 3h in sea water of PBS (phosphate buffered saline), pH 7.45, at room temperature. The tissue was then washed in 0.02% saponin in PBS for 30min, and then incubated in 1% NGS (normal goat serum) in PBS for 3h. The tissue was incubated overnight in the antiserum 146II, diluted 1:5000 in PBS at 4°C. The following day, the tissue was washed for 1h in 1% NGS in PBS, and then incubated in goat anti-rabbit (GAR) IgG linker (the Fc portion only) diluted 1:400 in PBS for 1h at room temperature. The tissue was again washed in 1% NGS in PBS, 3 times for 15min, at room temperature. The tissue was then incubated in rabbit PAP (peroxidase anti-peroxidase) IgG diluted 1:200 in TBS (tris buffered saline) for 1h at room temperature, and then washed in TBS (ph7.45) 3 times for 15 minutes. The reaction was developed in DAB-H_2O_2 (50

mg/100ml; 0.01 M Tris HCl and 0.01% H_2O_2) for 8 to 10 min. The DAB was replaced with PBS to stop the reaction. The tissue was then rinsed in a bicarbonate buffer 2 times for 15 min, and viewed for a reaction product under the light microscope. The tissue was osmicated in 1% OsO_4 in bicarbonate buffer for 1h at 4°C. The tissue was rinsed in bicarbonate buffer, dehyrated in a graded ethanol series to propylene oxide and embedded in epon:araldite. Thin sections were cut with a diamond knife, mounted on 300 mesh nickel grids, and viewed unstained in a Phillips 201 electron microscope. Numerous variations on this protocol were tried: the dilutions of all the antibodies and linkers were varied; instead of treating the tissue with saponin, or in conjuction with treating the tissue with saponin, the tissue was cryoprotected after fixation and then quick frozen in liquid nitrogen. Also, the tissue was sliced into 10μm sections after fixation with a Vibratome, to make the tissue thinner to allow for greater ease of penetration of the antibodies. Using a similar protocol, preembedding techniques using immunogold rather than PAP techniques were tried.

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